

UNIVERSIDADE DE LISBOA

Faculdade de Ciências

Departamento de Biologia Animal



**Stem cell-based reporter assays for the
identification of carcinogenic properties of
(non)-genotoxic chemicals**

Cátia Sofia Alão Gonçalves

DISSERTAÇÃO

MESTRADO EM BIOLOGIA HUMANA E AMBIENTE

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*Nothing in Biology Makes Sense
Except in the Light of Evolution"*

Theodosius Dobzhansk, 1973.

Resumo

Nos dias que correm, existem a geração contínua de novos produtos químicos, quer seja para o desenvolvimento de drogas, quer para aplicação na indústria alimentar, cosmética ou mesmo na agricultura. Portanto, antes que um composto possa ser colocado à venda no mercado, é necessário realizar vários testes, para que se possa excluir aquelas propriedades nocivas para a saúde humana, incluindo o potencial risco de cancro. No entanto devido ao crescente número de novos produtos químicos, existe a forte necessidade de encontrar ensaios *in vitro* que sejam rápidos em fornecer os resultados, baratos, relativamente fáceis de aplicar e ainda que apresentem uma taxa alta de sensibilidade de fiabilidade para avaliação do risco de cancro para o Homem.

O DNA é uma molécula complexa, que necessita de alguma integridade, para tal esta tem de ser protegida dos agentes ambientais, tais como a luz UV, produtos químicos provenientes de alimentos, medicamentos, ou mesmo gerada espontaneamente durante o metabolismo do DNA. Por esta razão, os organismos foram capazes de desenvolver vários mecanismos de defesa celular, incluindo as vias de reparação do DNA, os “checkpoint” do ciclo celular ou a indução da apoptose, de modo a proteger contra os efeitos tóxicos, mutagénicos de uma possível exposição.

As propriedades carcinogénicas dos químicos são frequentemente associados com o seu potencial genotóxico, ou seja, a capacidade de provocar lesões no DNA. Como resultado, estes químicos podem modificar a estrutura do DNA, levar à formação de ligações cruzadas, ductos, quebras simples e duplas da cadeia de DNA, bloquear a progressão do garfo de replicação ou os mecanismos de transcrição de um gene. A interferência da replicação do DNA ou da transcrição de um genes pode levar a mutações e rearranjos cromossómicos e, por vezes, pode levar a um evento oncogénico.

Muitos dos químicos carcinogénicos são capazes de levar a lesões do DNA e são “genotóxicos” no seu modo de acção. Existem, no entanto, um grupo de compostos carcinogénicos que induzem cancro na forma não-genotóxicos, ou seja, sem levar a

alterações do DNA, quer seja a nível do número de cromossoma quer seja a nível da estrutura.

O modo pelo qual os químicos carcinogénicos não-genotóxicos exercem a sua acção pode incluir os supressores imunológicos, proliferados de peroxissomas, agonistas dos receptores de hormonas, inibidores da fosfatase e os metalóides.

No entanto, ao contrário dos compostos carcinogénicos genotóxicos, o mecanismo pelo qual os carcinogénicos não-genotóxicos levam à carcinogénese ainda é desconhecido. Como tal, actualmente não existem ensaios *in vitro* disponíveis para estabelecer as propriedades potencialmente carcinogénicas dos compostos cancerígenos não-genotóxicos. Usualmente, para detectar agentes cancerígenos não-genotóxicos recorre-se a estratégias que utilizam ensaios baseados em parâmetros genotóxicos. O teste de Ames é um teste bacteriano que utiliza a mutagenicidade como parâmetros genotóxicos, mas pode-se também recorrer à genotoxicidade em células de mamíferos. No entanto, estes compostos não são detectados por estes ensaios, e acabam por passar despercebido. Logo, o ensaio mais utilizado para este caso de compostos químicos é recorrer a bioensaios para detectar a carcinogenicidade, usando roedores.

Logo, face ao aumento do número de composto e com vista à diminuição de bioensaios, realizámos este projecto que tinha como objectivo descrever a geração de um novo repórter baseado na utilização de GFP que permitia detectar as possíveis propriedades de compostos cancerígenos não-genotóxicos, que era específico para a resposta a proteínas mal enoveladas (UPR, do inglês: *Unfolded Protein Response*)

Para que se pudesse desenvolver estes repórteres baseados em GFP, realizámos um “genome-wide transcription profiling” utilizando mES que foram previamente expostas a diversos agentes cancerígenos não-genotóxicos.

Após análise dos “genome-wide transcription profiling”, foi possível seleccionar quatro genes biomarcadores, Armet, Derl3, Dnajc3 e Ddit3, que eram induzidos após exposição a supressores imunológicos e a metalóides. Recorrendo ao qRT-PCR confirmámos esta indução, e verificámos que os genes biomarcadores Armet, Derl3 e Dnajc3 eram induzidos após exposição com o supressor imunológico ciclosporina A, e o gene biomarcador Ddit3 era induzido após exposição ao metalóide arsenito de sódio.

Para que os genes biomarcadores que seleccionámos pudessem ser utilizados como repórteres baseados em GFP, recorreremos à técnica de recombinação de cromossoma artificial bacteriano (BAC, do inglês: *bacterial artificial chromosome*). Esta técnica consiste em gerar linhas celulares que expressam uma proteína de fusão de GFP e, portanto, é possível determinar facilmente indução do gene pela quantidade de

fluorescência emitida pela proteína de fusão de GFP modificada após o tratamento com um determinado composto.

Uma vez que os genes biomarcadores, Armet-GFP, Der13-GFP, Dnajc3-GFP e Ddit3-GFP, se encontraram funcionais, estes foram expostos a cancerígenos não-genotóxicos, que incluía a: cisclosporina A; o arsenito de sódio; e a tunicamicina, e a carcinogéneos genotóxicos, nomeadamente a cisplatina, a doxorubicina e a peróxido de hidrogénio. Após análise da citometria de fluxo, verificámos que os nossos genes biomarcadores não eram induzidos por químicos carcinogéneos genotóxicos, mas que por sua vez eram fortemente induzidos quer por a cisclosporina A e arsenito de sódio, mas também por tunicamicina, que nos levou a afirmar que estes biomarcadores eram específicos para o UPR e que o supressor imunológico cisclosporina A e o ao metalóide arsenito de sódio estão envolvidos no UPR.

Uma vez desenvolvidos os repórteres GFP, o próximo passo era testar a especificidade e sensibilidade. Para tal, validámos, juntamente com os genes biomarcadores para os compostos cancerígenos não-genotóxicos, uns repórteres GFP desenvolvidos anteriormente para os compostos carcinogéneos genotóxicos. Os repórteres GFP foram expostos a 53 químicos propostos pela ECVAM, que inclui compostos carcinogéneos genotóxicos, cancerígenos não-genotóxicos e compostos não-carcinogénicos. Após análise dos dados, observámos que tínhamos desenvolvido repórteres GFP com diferentes sensibilidades, uns eram capazes de detectar compostos carcinogéneos genotóxicos que induziam lesões genotóxicas ou stress oxidativo, e outros que eram capazes de detectar compostos cancerígenos não-genotóxicos que detectavam compostos que induziam lesões nas proteínas.

Para que pudéssemos correlacionar a activação dos repórteres GFP com um fim biológico, decidimos recorrer a um ensaio *in vitro* vulgarmente utilizado para detectar genotoxicidade. Recorremos à utilização do ensaio do cometa, visto este ser um ensaio que é amplamente utilizado para avaliar lesões no DNA. No entanto, este ensaio ainda não tinha sido descrito para as mES. Começamos por otimizar o ensaio do cometa e concluímos que este não tinha a capacidade de detectar compostos cancerígenos não-genotóxicos, e para além de que mesmo tendo a capacidade de detectar compostos carcinogéneos genotóxicos, não eram informativo sobre o tipo de lesão que induzia, quer fosse lesões no DNA ou dano oxidativo.

Em suma, podemos concluir que ao usar a combinação de diferentes linhagens celulares de repórteres baseados em GFP, somos capazes de discriminar entre compostos que induzem principalmente lesões genotóxicas, stress oxidativo ou lesões nas proteínas

Com este estudo fomos ainda capazes de demonstrar que o ensaio do cometa, um ensaio tradicionalmente utilizado *in vitro* para detectar propriedades genotóxicas de compostos, não tem a mesma sensibilidade e especificidade que os repórteres GFP que desenvolvemos, na medida em que o ensaio do cometa não permitiu detectar compostos carcinogénicos não-genotóxicos e não forneceu qualquer informação sobre o tipo de lesão causada pelos compostos carcinogénicos genotóxicos.

Com este estudo, podemos afirmar que desenvolvemos um ensaio *in vitro*, que é altamente sensível e específico, que pode fornecer informações sobre a toxicidade relativa de produtos químicos, e que ao mesmo tempo é rápido e tem elevada fiabilidade para avaliação do risco de cancro para o Homem e que permite de certa forma levar a redução de biosensaís.

Palavras-chave: Repórteres-GFP, carcinogénicos não-genotóxicos, genotoxicidade, UPR.

Abstract

There is a continuous generation of new chemicals for drug development or applications in food industry, cosmetics and agriculture. Before compounds are allowed on the market, they first require testing to exclude hazardous properties on human health, including potential cancer risk. Due to the increasing number of new chemicals there is a strong demand for rapid, easy to use high-throughput *in vitro* assays for human cancer risk assessment. The induction of cancer is strongly associated with DNA damage and mutations upon exposure to genotoxic compounds. However, there are many non-genotoxic (NGTX) compounds that have been shown to be carcinogenic. The mechanisms by which these non-genotoxic carcinogens (NGTXC) induce cancer is often unclear. Here we describe the development of four different mES reporter cell lines for the detection of possible carcinogenic properties of non-genotoxic chemicals. The GFP-based reporter cell lines are based on the activation of biomarker genes that are predictive for exposure to specific NGTXC. By genome transcription profiling of mES and qRT-PCR we identified genes that were transcriptionally activated upon exposure to NGTXC. Four genes (Armet, Dnajc3, Derl3 and Ddit3) were induced by the immune suppressors CsA and Fk506 and by the metalloid NaAsO₂. These genes were generated by BAC recombineering, were GFP-reporters genes were stably integrated in mES cells. These biomarker genes have previously been associated with the induction of the unfolded protein response (UPR), suggesting the UPR is a mode of action of CsA and NaAsO₂. These GFP-reporters cell lines are specifically activated upon exposure to compounds that induce protein damage. A panel of different mES reporter cell lines will allow identification of the potential carcinogenic properties of non-genotoxic chemicals and in addition can detect protein damage.

Keywords: GFP-reporter, non-genotoxic carcinogens, genotoxicity, UPR

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Abbreviations

CDC25	Cell division cycle 25
cDNA	Complementary deoxyribonucleic acid
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
GADD45 α	Growth arrest and DNA damage inducible alpha
GFP	Green fluorescent protein
G418	Geneticin
Hprt	Hypoxanthine Phosphoribosyltransferase 1
mES	Mouse embryonic stem cells
MRE11	Meiotic recombination 11
NaOH	Sodium hydroxide
NBS1	Nijmegen Breakage Syndrome 1
NF-kB	Nuclear Factor Kappa Beta
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
qRT-PCR	Quantitative reverse transcriptase PCR
RNA	RiboNucleic Acid
ROS	Reactive oxygen species
siRNA	Small interfering RNA
SD	Standard deviation
TBE	Tris/Borate/EDTA
TE	Tris-EDTA
UV	Ultraviolet
YWHAD	Protein Kinase C Inhibitor Protein-1

Chapter 1

Introduction

Every year, industry developed compounds for a broad range of applications. However, these compounds can have harmful properties on human health, since they can interact with different cellular structures. Compound exposure can induce mutation, affect genome stability, result in ROS formation, contribute to aging processes and can potentiate genetic diseases and cancer risk [1,2] (Fig. 1).

According to estimates of the International Agency for Research on Cancer (IARC), cancer is a leading cause of death and was responsible, in 2008, for 7.6 million deaths worldwide [3]. Therefore, to determine the future risk of cancer it is important to assess the hazard of compounds, as well as developing new techniques to assess the risk.

1.1. Genotoxic carcinogens

DNA is a complex organic molecule responsible for the genetic information of cell bodies. Thus, for the information to be successfully transmitted from generation to generation, DNA integrity must be maintained [4]. To maintain genomic integrity, DNA must be protected from damage induced by environmental agents, such as UV light, chemicals from food, drugs, or generated spontaneously during DNA metabolism [5]. For this reason, organisms have evolved various cellular defence mechanisms, including DNA repair pathways, cell cycle checkpoints or induction of apoptosis, in order to protect against the toxic, mutagenic, and possible oncogenic consequences of exposure [6] (Fig. 1).

The carcinogenic properties of compounds are often associated with their genotoxic potential, i.e., the ability to cause DNA damage. As a result, these compounds can modify the DNA structure, lead to the formation of cross-links, bulky DNA adducts, single- and double strand breaks (SSBs and DSBs) and block progression of the DNA

replication or gene transcription machineries. Interference with DNA replication or gene transcription can lead to mutations and chromosomal rearrangements and sometimes can result in an oncogenic event [7].

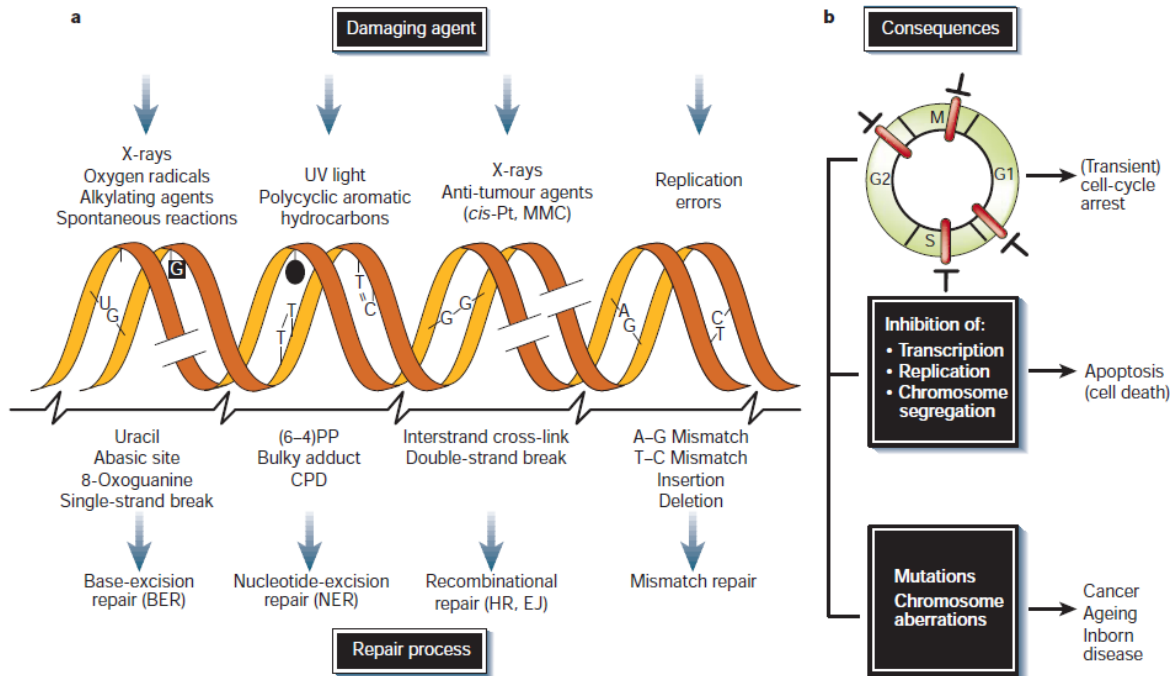


Fig. 1. DNA damage agents, repair process and consequences. a) DNA damaging agents (top); DNA lesions induced by these agents (middle); DNA repairs systems responsible for the removal of the lesions (bottom). b) DNA damage effects on cell-cycle progression, with the arrest in the G1, S, G2 and M phases (top); and on DNA metabolism (middle); Long-term consequences of DNA injury (bottom) (adapted from Hoeijmakers [5]).

1.2. DNA repair systems

In order for the DSB repair mechanisms to be successfully carried out, eukaryotic cells have developed a cellular mechanisms that regulate the recruitment of DNA repair factors to sites of DNA damage, activate those factors, and coordinate the choice of the pathways to employ for efficient DNA repair [8].

A variety of damage can occur in the DNA, thus, to respond to DNA damage, repair mechanisms specific for many types of lesion have evolved directly to a specific type of damage. Bulky distortions in the DNA helix are detected and repaired by nucleotide excision repair (NER) [9,10], while base excision repair (BER) repairs smaller lesions such as deamination of a base through excision of the damaged base [11]. Mismatched DNA bases are replaced with correct bases by mismatch repair (MMR) [5,12].

SSBs are repaired by single-strand break repair (SSBR), whereas DSBs are processed either by homologous recombination (HR) or non-homologous end joining (NHEJ). HR always takes place during replication as it uses the information of the sister chromatid as a template for repair, to restore the genomic sequence of the broken DNA ends. While NHEJ does not make use of a sister chromatid, it merely joints and ligates the broken ends independently of the previous DNA sequence. Thus, NHEJ can take place at any time point in the cell-cycle and promotes the potentially inaccurate rejoining of DSBs [13].

However, the cell is not always able to repair the damage and therefore the cell might try to bypass the damage by different mechanisms and continue replication. During replication the cell might bypass the damage via two routes either by damage avoidance or by translesion synthesis (TLS) [2,6]. Damage avoidance uses the sister chromatid as template for further replication and thus is error free. Whereas translation synthesis uses a different polymerase than can more easily read over the damage. However, this polymerase has greatly reduced template specificity, therefore this polymerase has a high error rate and often incorporates incorrect bases [6]. A high error rate can ultimately lead to incorporation of mutations into the genome and can therefore be a cause of carcinogen.

Each pathway consists of numerous proteins forming a cascade in order to repair the damage as accurate as possible. The activation of cell cycle checkpoints stops the proliferating cell in its cell cycle progression in order to give time to the DNA damage repair machinery to repair the lesion [14,15]. However, when the repair process fails, the cell cycle can be blocked permanently. Therefore, the most efficient mechanism to protect cells against the genotoxicity of a compound is apoptosis. By apoptosis cells that express high levels of DNA damage are removed from the exposed tissue, preventing mutagenesis of the cells [6]. Thereby, all the mechanisms contribute to prevent incorrect genetic information from being passed onto the progeny

1.2.1. DNA damage response (DDR)

The DNA damage response is a kinase-dependent signalling pathways, composed by the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), that have the ability to sense DNA damage and transduce this information to the cell to influence cellular response to DNA damage [1,16].

Both kinases promote cell cycle checkpoint activation and DNA repair systems in response to genotoxic agents. ATM is recruited to DNA double-strand breaks (DSBs)

by its regulator the MRN (MRE11, Rad50-NBS1) and is required for efficient DSB repair [7,17]. The ATR kinase with its regulator ATRIP (ATR-interacting protein) senses single-strand DNA (ssDNA) and is activated in response to stalling of DNA replication and is recruited to stalled replication forks. Activation of ATM results in phosphorylation of the Chk2, whereas ATR activates the Chk1. Both Chk1 and Chk2 inhibit CDC25 phosphatase and the p53 tumor suppressor, thereby blocking activation of the cyclin-dependent kinases, inhibiting cell cycle progression, and activating the induction of apoptosis [17,18].

1.3. Current Methods for (Geno) toxicity Testing

Due to the fast development of new compounds and prior to their introduction on the market, it is necessary to develop an assay that is able to assess the potential genotoxic or other deleterious properties of newly developed compounds. It's crucial for industries, that the assays are fast, cheap and predictive for animals and humans health risk, as well as the environment. High sensitivity and specificity, together with few false-positives or negatives are the features for a good predictive assay [19]. Furthermore, an assay should also provide information about the mode of toxicity of a compound and be able to predict adverse health effects.

There are various validated *in vitro* and *in vivo* assay to test the potential cancer hazard of a compound, as well as to detect biological endpoints of exposure. The assays have the ability to measures the DNA damage, mutation or chromosomal aberrations [20].

One of the classic tests for genotoxicity is the Ames test. The Ames test is a bacterial assay that is able to assess the mutagenic potential of a compound by means of the reversion of mutations in a modified *Salmonella Typhimurium* [7]. The bacterium has a mutation in the histidine gene, making it dependant on a histidine for growth. A possible mutagen compound is considered a mutagen when it is able to mutate the bacterium in a way that it loses its dependence on histidine [20]. However, the Ames test has a relative low sensitivity and therefore often fails in identifying genotoxic properties [21]. Other tests for bacterial mutagenicity, is the SOS/umu-test. This test makes use of the SOS response, in which the cell cycle is arrested and DNA repair is induced. This response is activated upon exposure to genotoxic compound [21].

To establish genotoxicity in eukaryotes, several yeast-based assays have been developed. The DELL assay is based on the revision of mutation in a modified *Saccharomyces cerevisiae* strains containing a mutation in the *his3* gene. The

GreenScreen GS assay contains a GFP coupled to the Rad54 gene promoter. This assay monitors the activation of the DNA damage response, by the activation of Rad54 gene expression, when induced upon exposure to various DNA damaging agent [21].

Nevertheless, to assess the risk to human is crucial to use genotoxicity tests based on mammalian cells, however has proven to be much more challenging. The comet assay (single cell gel electrophoresis) detects DNA SSB and DSB, alkaline labile sites and excision repair processes in individual cells. The Comet assay detects DNA breaks via a protocol of cell lysis and electrophoresis under low voltage allowing migration of damaged DNA, if DNA damage has occurred the electrophoresed DNA fragments migrate towards the anode, and appear as a diffuse tail behind the nucleus, producing a shape resembling a comet [22].

The Mouse lymphoma TK assay (MLA) is able to detect mutagenic and clastogenic events at the thymidine kinase (tk) locus of L5178Y mouse lymphoma tk (+/-) cells. The assay makes use of the acquirement of resistance to trifluorothymidine and uses the number and size of cultured clones as read out [23].

The GreenScreen HC assay and the ToxTracker assay are newly developed assays that make use of cellular signalling pathways. The principle of these two tests is based on the activation of biomarker genes involved in the pathways activated upon DNA damage¹. The GreenScreen HC makes use of human lymphoblastoid TK6 cells and is based on activation of the *GADD45α* gene, which is a known target of the *p53* tumor suppressor. However, also other signalling pathways, *Nrf2* and *Nf-κB*, transcription factor that play a role in inflammation, which have been associated with *Gadd54α* induction [7,24].

The ToxTracker assay makes use of transcriptional changes after cell treatment. The assay uses a panel of three GFP-based mES reporter cell lines whose genes are induced after treatment with genotoxic carcinogen compounds. The biomarker genes are the *Bsc12* (Bernardelli-Seip congenital lipotrophy 2), the *Srxn1* (sulfiredoxin 1) and the *Btg2* (B-cell translocation gene 2) [1]. *Bsc12-GFP* is specifically induced upon exposure to direct DNA damaging agents, the GFP reporter gene is activated in response to DNA replication inhibition and depends on ATR-Chk1 signalling from stalled replication forks [1,7]. The *Srxn1-GFP* reporter gene is strongly activated upon cellular oxidative stress and directly controlled by the *Nrf2* antioxidant pathway [1,7]. The *Btg2-GFP* reporter is a *p53*-depend and responsive to DNA-damaging agents and pro-oxidant [7,21]. Together these different cell lines are able to discriminate between compounds that primarily induce genotoxic or oxidative stress [7].

1.4. Non-genotoxic carcinogens

Most chemical carcinogens are able to induce DNA damage and are ‘genotoxic’ in their carcinogenic mode of action. There is, however, a group of carcinogens that induce cancer in a non-genotoxic manner, without altering DNA, chromosome number or structure.

Among the processes that non-genotoxic carcinogens have been shown to include immune suppressors, peroxisome proliferators, hormone receptor agonists, phosphatase inhibitors and metalloids [7,25]. However, unlike genotoxic carcinogens which share a unifying characteristic that is genotoxicity, non-genotoxic carcinogens can have diverse modes of action which are, for the most part, tissue and species specific [25], and it’s unclear the exact mechanism by which exposure of these compounds contributes to carcinogenesis [19]. Albeit, the monitoring of non-genotoxic carcinogens is difficult and there are no general markers suitable for all compound classes [19].

Non-genotoxic carcinogens are known to interact with the function of peroxisome proliferators. Peroxisome proliferators are normally involved in mitochondrial and peroxisomal fatty-acid uptake and beta-oxidation. The binding of peroxisome proliferator receptors (PPAR α) by non-genotoxic compounds can result in inhibition of apoptosis and in cell proliferation [26]. Cell proliferation is one of the characteristics of carcinogenesis and can also be dependent on the binding of estrogenic substances to intracellular estrogen receptors [27]. Have been previously described that some non-genotoxic carcinogens are estrogen agonists, and also have been implied in the activation of PKC via a target site or receptor [28].

Some non-genotoxic carcinogens have been proven to be immune suppressors [29]. Immune suppression is also known to play a role in carcinogenesis. The immune system attack harmful cells, however when the immune suppressor are administered the immune reactions are suppressed [29], making occurrence and growth of tumors more likely. Nevertheless, in most cases a link between the mode of action of a compound and its carcinogenic effects has not been established [7].

In contrast to genotoxic carcinogens, there are currently no validated *in vitro* assays available to establish potentially carcinogenic properties for the non-genotoxic compounds [27]. Usually, to detect non-genotoxic carcinogens the used strategy are based on genotoxic endpoints including the Ames mutagenicity test in bacteria, genotoxicity in mammalian cells and germ cell mutagenicity tests, nevertheless non-genotoxic carcinogens are negative in these tests and thus go undetected [25]. Therefore, the traditional assay to detect carcinogenicity is to use rodent 2-year cancer

bioassay [30], however there is a new European policy which try to reduce the number of cancer bioassays [25].

It's crucial to develop an assay that is fast, cheap and allows to reduce the number of animals that are used in the bioassays. However, the lack of assays is due to the diversity of effects elicited by different non-genotoxic carcinogens on the cells physiology. This diversity of effects makes the development of assays to assess carcinogenic effects highly challenging.

A way to solve the problem of lack of assays is to select biomarkers for the difference modes of action the non-genotoxic carcinogens and to assess the gene expression of a cell after treatment with different groups of chemicals [31]. Thus, gene expression profiling might be able to distinct genes specific for a certain response pathway activated after treatment. When a gene is selective for a certain class of compounds it can be used as a biomarker gene for distinction between classes of compounds, like genotoxic carcinogens and non-genotoxic carcinogens [31].

Objectives

The aim of this project was to describe the generation of a novel GFP-based reporter for the detection of possible carcinogenic properties of non-genotoxic compounds, that were specific for the unfolded protein response (UPR).

A systematic genome-wide transcription profiling in mES cells was previously performed on cells exposed to different groups of non-genotoxic carcinogens. Based on the genome-wide transcription profiling, we selected a set of putative biomarker genes for exposure to different classes of NGTXC. We selected three biomarker genes that were specifically induced upon exposure to different immune suppressors and one biomarker genes upon exposure to metalloids. The GFP-based reporter cell lines were generated with BAC recombinational techniques. This technique generates cell lines expressing a GFP fusion protein and therefore functions as a high-throughput method for the examination of the proteins function and induction inside the cell [31]. The GFP biomarker genes were preferentially activated upon exposure to compounds that induce the UPR.

Besides, we validated GFP-based reporters that were developed for NGTXC together with GFP reporters that were developed for detection of carcinogenic properties of genotoxic carcinogens compounds. In order to correlate the GFP-reporter

activation with a biological endpoint we used an *in vitro* assay to establish reporter activation. We use the comet assay, since is an assay that is widely used to measure DNA damage [22]. We optimized the comet assay for the mES, since it is not yet described for this cells type.

Chapter 2

Material and Methods

Embryonic stem cell culture and treatments

mES cells were cultured as described previously [1]. C57/Bl6 B4418 wild-type mES cells were cultured in Knockout DMEM (Gibco) which contained 10% fetal bovine serum, 2mM GlutaMAX (Gibco), 1mM sodium pyruvate (Gibco), 100 μ M β -mercaptoethanol (Gibco), and leukemia inhibitory factor (LIF). mES cells were culture on irradiated primary mouse embryonic fibroblasts as feeders according to the described protocol [21].

mES cells were seeded 24hrs prior to compounds exposure on gelatin-coated 96-well plates in the absence of feeder cells in buffalo rat liver cell (BRL)-conditioned ES cell medium, as has previously been described¹. The mES cells were treated with five increasing concentrations per compound that were prepared in DMSO or PBS and added directly to the culture medium.

For treatment that did not required metabolic activation, the cells were continuously exposed for 24hrs. 24hr after cells were washed with PBS, trypsinized and PBS+2% serum was added before FACS analysis.

Gene expression profiling

Induction of the genes selected during the previously performed genome wide transcription profiling was validated using qRT-PCR. Cells were on gelatin-coated 6-well plates, as described [1].

Detailed protocol for the RNA isolation and cDNA synthesis can be found in supplementary method 2. Total RNA was isolated after 8 or 16 h treatment using TRIzol (Invitrogen) and the total RNA concentration was determined by

spectrophotometer NanoDrop (Isogen ND-1000). cDNA was synthesized using oligo (dT) 12–18 primers and SuperScript Reverse Transcriptase III (Invitrogen) according to the manufacturer's protocol. Gene expression was determined using specific primers with SYBR Green Supermix (BIO RAD) on a Real Time System C1000 Touch Thermal Cycler (BIO RAD). Expression was normalized using expression of the YWHAD and Hprt genes.

Generation of GFP reporter cell lines

Selection of biomarker genes that were used to create GFP-based reporters was based on the genome wide transcription profiling that has been described [21]. The genes: Bhlhb2, Grasp, Ddit3, Atf3 and Krt8 were selected to generate the GFP reporters. The GFP reporters were generated by BAC recombineering as described by Poser *et al.* [32] and Hendriks *et al.* [1]. See supplementary method 1 for a detailed protocol for BAC recombineering.

The bacterial artificial chromosome (BAC) containing the biomarker gene were selected using mouse BAC finder and purchased from BACPAC. We order the BAC RP24-228C19 for the biomarker gene Bhlhb2, BAC PR24-312P21 for Grasp, BAC RP24-175A16 for Ddit3, BAC RP24-318C6 for Atf3 and the BAC RP24-152H23 for Krt8.

The biomarker gene were modified with a C-terminal GFP green fluorescent marker [32] using the Quick & Easy BAC modification Kit (Gene Bridges).

BAC strains were transformed with the pRedE/T plasmid followed by Tet selection. PCR fragments encoding a GFP-ires-Neo reporter cassette were generated using primers that contain an additional 50 nucleotides sequence homologous to the 3' sequence of the biomarker gene on the BAC, and were transformed into the BAC strain, as has been described by Hendriks *et al.* [1]. BAC with the PCR fragment were grown on kanamycin plates, the clones were analysed for proper integration of the GFP cassette and the modified BAC were isolated with the Nucleobond PC100 DNA isolation kit (Macherey-Nagel).

The BACs were analysed for proper integration by PCR and confirmed by sequence analysis. 6×10^5 mES cells were seeded on gelatin-coated culture dishes 24h prior to transfection, in BRL-conditioned ES cell medium in the absence of feeder cells. The mES cells were transfected using Lipofectamine 2000 (Invitrogen), as described by Poser *et al.* [32]. The next day the cells were washed with PBS and BRL-conditioned

ES cell medium was added. After 24h G418 was added to the medium, to select for Neomycin resistance. Two weeks after transfection, monoclonal cell lines were picked and grown under constant selection of G418. Monoclonal mES cell lines were selected based on GFP expression after exposure to either clofibrate or sodium arsenite according to the non-genotoxic compound that were selected. GFP expression was determined by flow cytometry

Detection of GFP expression

To test the GFP reporter expression induced by a compound, the cells were cultured on gelatin-coated 96-wells plates, and 24h after were treated with five different concentrations. The compounds were prepared in DMSO or PBS and diluted in BRL-condition ES cell medium.

GFP reporter expression was determined by flow cytometry (Guava easyCyte HT, EMD Millipore) as the mean fluorescence intensity of 5000 intact cells. Cell viability was also assessed by FACS.

Validation of GFP reporter cell lines

The GFP-based reporters cell lines were validated with 53 genotoxic and non-genotoxic compounds, belonging to a list of chemicals suggested for validation in vitro genotoxicity test assay by the European Centre for Validation of Alternative Methods (ECVAM) [33] (table 4).

Compound concentrations were based on cytotoxicity, which had been previously described¹. 10mM was the highest concentration that was used [30], when the compound did not affect the cell viability. Induction of GFP-based reporters was assessed after 24h exposure by the flow cytometer (Guava easyCyte HT, EMD Millipore).

We considered an activation of a reporter cell line positive when the exposure to a compound resulted in > 1.5-fold induction of GFP expression that is at least five times greater than the SD in background fluorescence in mock-exposed cells, described by Hendriks *et al.* [1]. Activation of a reporter cells line is based on the mean of at least three independent experiments, treated with five different concentrations compounds.

Comet assay

The comet assay was performed under alkaline and neutral conditions according to Olive & Banáth [34], with some minor modifications, that are described in detail in supplemental method 3.

The mES cells were seeded as described previously, with different compounds on gelatin-coated 6-well plate in BRL-conditioned medium, for 15min, 30min, 1, 2, 4, 6 and 8hrs according to the administered compound (supplemental table 1).

Cells suspensions ($3,5 \times 10^5$ cells) were chilled on ice and centrifuged at 1000rpm for 5min. After the supernatant was removed, the cells were re-suspended in a solution of 0.8% of low melting point agarose (LMA) (NuSieve GTG) dissolved in PBS. 100 μ L of cell suspension was spread over a microscope slide, previously coated with 1.5% agarose (Roche) and immediately covered with a cover glass to make a microgel on the slide. The slides were kept at low temperature for at least 10min to allow the agarose to solidify. The cover glass was removed and the slides were immersed in cold Lysis Solution (Trevigen, Sanbio). The slides were 1h on cold lysis solution in the dark. Slides were removed from lysis solution, and then, for alkaline condition, the slides were submerged for 30min in unwinding solution (10M NaOH and 0.5M EDTA, pH ~13). The slides were placed on a horizontal electrophoresis unit, and the DNA was allowed to unwind for 30 min in freshly prepared alkaline electrophoresis buffer. Electrophoresis was conducted at 27 V and 300 mA. All technical steps were conducted at low temperature (4°C) to avoid additional DNA damage. After electrophoresis, the slides were gently removed from the electrophoresis unit, and were placed three times in neutralization buffer containing 0.4M Tris at pH 7.5, 5min and dipped in 70% alcohol for 30min. The slides were air dried and stored at room temperature until analysis.

For neutral condition, after the lysis step, cells were washed twice with neutral solution (1xTBE), 5min each. Slides were placed on electrophoresis unit, and the DNA was allowed to migrate for 20 min in the buffer, at low temperature. After electrophoresis, the slides were washed two times with bi-distilled water during 5min each, and rinsed with 70% alcohol for 30min. The slides were air dried and stored at room temperature until analysis.

Slides were stained with SYBR Gold nucleic acid gel stain (Molecular Probes-Invitrogen), TE buffer and β -mercaptoethanol, over 30min. After 30min, the slides were washed with TE and covered with a cover glass. They were allowed to dry and then analysed.

For scoring the DNA damage, the samples were examined under a 20x objective on a fluorescence microscope using FITC filters (490nm excitation/520nm emission) (Zeiss AXIO, Germany). The images of the comets were analysed using the CometScore software (TriTek, AutoComet.com). The parameter used to measure DNA damage in the cells was the tail moment. The tail moment is the product of 'tail length' and '%DNA in tail / 100' (Fig. 2).

At least fifty randomly selected cells per slide were scored for the cell culture experiments. The mean of the tail moment for each experiment was calculated. DNA damage was considered positive when exposure to a compound resulted in an increase of more than 0.0912 in tail moment, which is five times higher than the SD in the untreated cells.

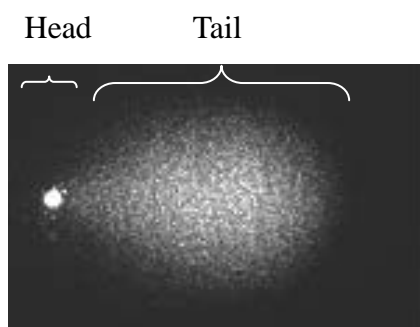


Fig. 2. Representation of a comet. Comet of 30min 500 μ M of hydrogen peroxide.

Chapter 3

Results

3.1. Reporters for non-genotoxic carcinogens

3.1.1. Identification of biomarkers genes

In order to identify biomarker genes for non-genotoxic carcinogens, mES were exposed to 23 non-genotoxic (NGTX) carcinogen and non-genotoxic noncarcinogen (table 1) for 8h, and a genome wide transcription profiling of mES cells was performed. Table 1 shows which compounds and concentration were used to select biomarker genes.

Table 1

Chemicals and concentrations used for exposure of wt mES cell line. The table shows two different classes of compounds subdivided into different groups and their biological relevance, abbreviation and the concentrations that were tested with a genome wide transcription profiling and the concentration tested with the qRT-PCR.

Class	Biological relevance	Treatment	Abbreviation	Conc. (μ M) Array	Conc. (μ M) qRT-PCR
<i>NGTX carcinogens</i>					
	Immune suppressor	Cyclosporin A	CsA	10	50
		Tacrolimus	Fk506	10	25
	PPAR α ligand	Wyeth-14,643	WY	250	250
		Clofibrate	CLO	100	400
	CAR ligand	Phenobarbital	PB	3000	
		1,4-bis[2-(3,5-dichloro pyridyloxy)]benzene	TCPOBOP	5	
	Organochloride pesticides	β -Hexachlorocyclohexane	β -HCH	50	
		Heptachlor Epoxide	Hept. Epox	5	
	Ahr ligand	2,3,7,8-Tetrachlorodibenzo-p-dioxin	TCDD	0.1	
		Aroclor 1254	Aroclor 1254	25	
	PKC activator	Phorbol 12-myristate 13 acetate	PMA	1	
		Phorbol 12,13-dibutyrate	PDBU	1	
	Ozone depletion	Carbon Tetrachloride	CT	3	
		1,1,1-trichloroethane	TCE	3	
	PP2A inhibitor	Okadaic Acid	OA	4×10^{-3}	
		Calyculin A	CLA	0.5×10^{-3}	
	Metalloid	Sodium Arsenite	NaAsO ₂	2	5
		Lead Acetate	PbAc	10	100
	Er α activator	Diethylstilbestrol	DES	2.5	
<i>NGTX noncarcinogens</i>					
	PPAR α ligand	Diisodecyl phthalate	DIDP	2000	
	Tributyltin oxide	TBTO	0.25		
	Sugar Alcohol	D-mannitol	Man	2000	
	Ahr/Er α /SXR/PXR activator	Bisphenol A	BPA	80	

After data normalization and statistical analysis of the genome wide transcription profiling, genes that showed specific increase in expression after treatment with non-genotoxic carcinogens were ranked based on the fold change in gene expression (data not shown). The biomarker genes that showed the highest induction belonged to immune suppressor, PPAR α and metalloids, and are grouped by classes in table 2.

Table 2

Biomarker genes that were selected by genome wide transcription profiling of mES. The biomarker genes were selected after treatment with different classes of non-genotoxic carcinogens, to select the biomarker for the immune suppressor cells were exposed to CsA and Fk506, to PPAR α ligand mES were exposed to WY and CLO and to metalloids mES were exposed to NaAsO₂ and PbAc.

Immune suppressor	PPAR α ligand	Metalloid
Armet	Kl11	Ddit3
Sdf211	Cib4	Gpr124
Dnajc3	Makp4	Grb10
H47	Hebp1	Atf3
Derl3	Cln5	Krt8
Pdia4	Bhlhb2	Slc40a1
	Grasp	Sympo2
	Camk2n	
	Ceacam1	

To confirm the induction of biomarker genes that were identified by genome wide transcription profiling we analysed the expression of the 22 genes by qRT-PCR. For this, wild-type mES cells were exposed for 8 and 16h to CsA and Fk506 (immune suppressor), WY and CLO (PPAR α ligands), NaAsO₂ and PbAc (metalloids) and to cisplatin, a genotoxic agent. After qRT-PCR analysis, only the biomarker genes that showed the highest induction were chosen.

For the different biomarker genes that were selected for the immune suppressor we selected Armet, Derl3, Dnajc3 and H47 as biomarker genes, since were the ones that showed the higher fold change after treatment (Fig. 3). These four genes when exposed to CsA showed a higher induction, compared with exposure of Fk506, and none of the genes showed induction when exposed to the genotoxic agent. In contrast, genes Sdf211 and Pdia4 showed no change when exposed to the treatment.

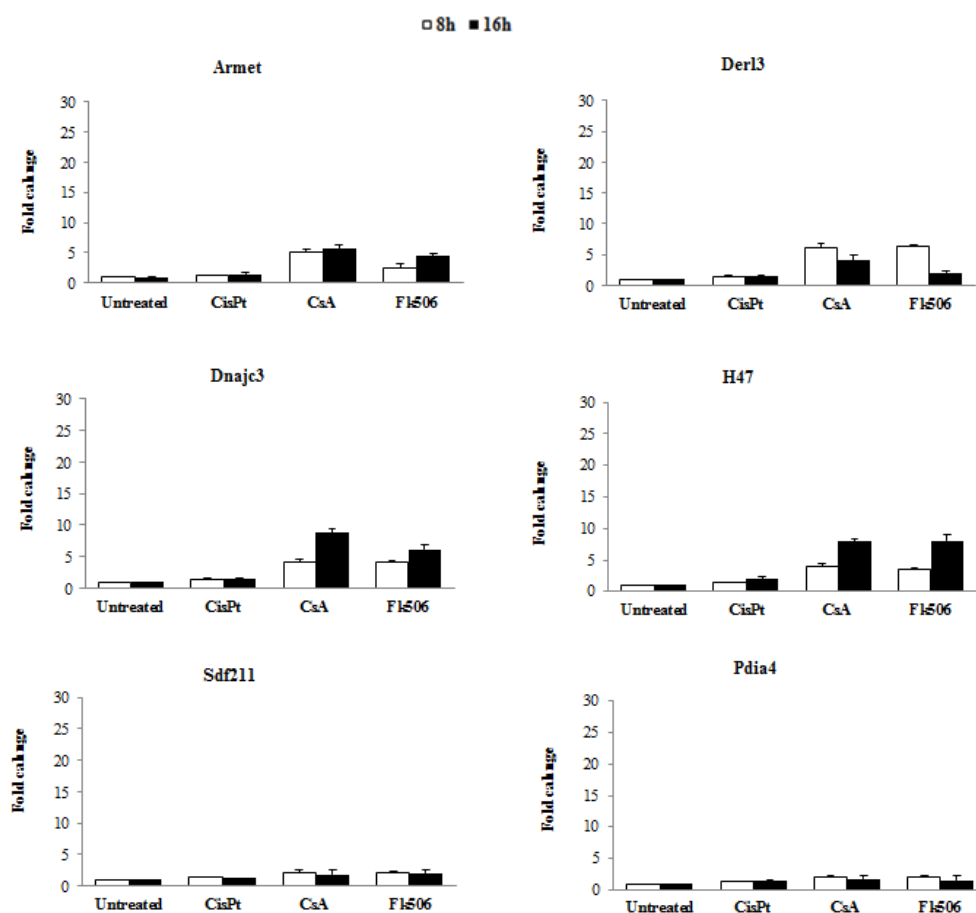


Fig. 3. Expression of possible biomarker genes for immune suppressor by qRT-PCR. Genes were tested on their expression with a qRT-PCR after 8 and 16h exposure to CisPt 5 μ M, CsA 50 μ M and Fk506 25 μ M.

Of the various biomarkers genes that were selected for PPAR α ligand, we selected Bhlhb2 and Grasp, because were the only two genes that showed the higher induction after treatment (Fig. 4).

The biomarker gene Bhlhb2 was selected based on gene induction after 8h treatment to CLO and did not show induction after exposure to cisplatin. Grasp was induced for both the peroxisome proliferators (WY and CLO), but only after 16h treatment. When exposed to cisplatin showed a slight induction, but was not significant. We exposed the mES to CsA, however these genes were not induced by this compound, thus indicating some specificity.

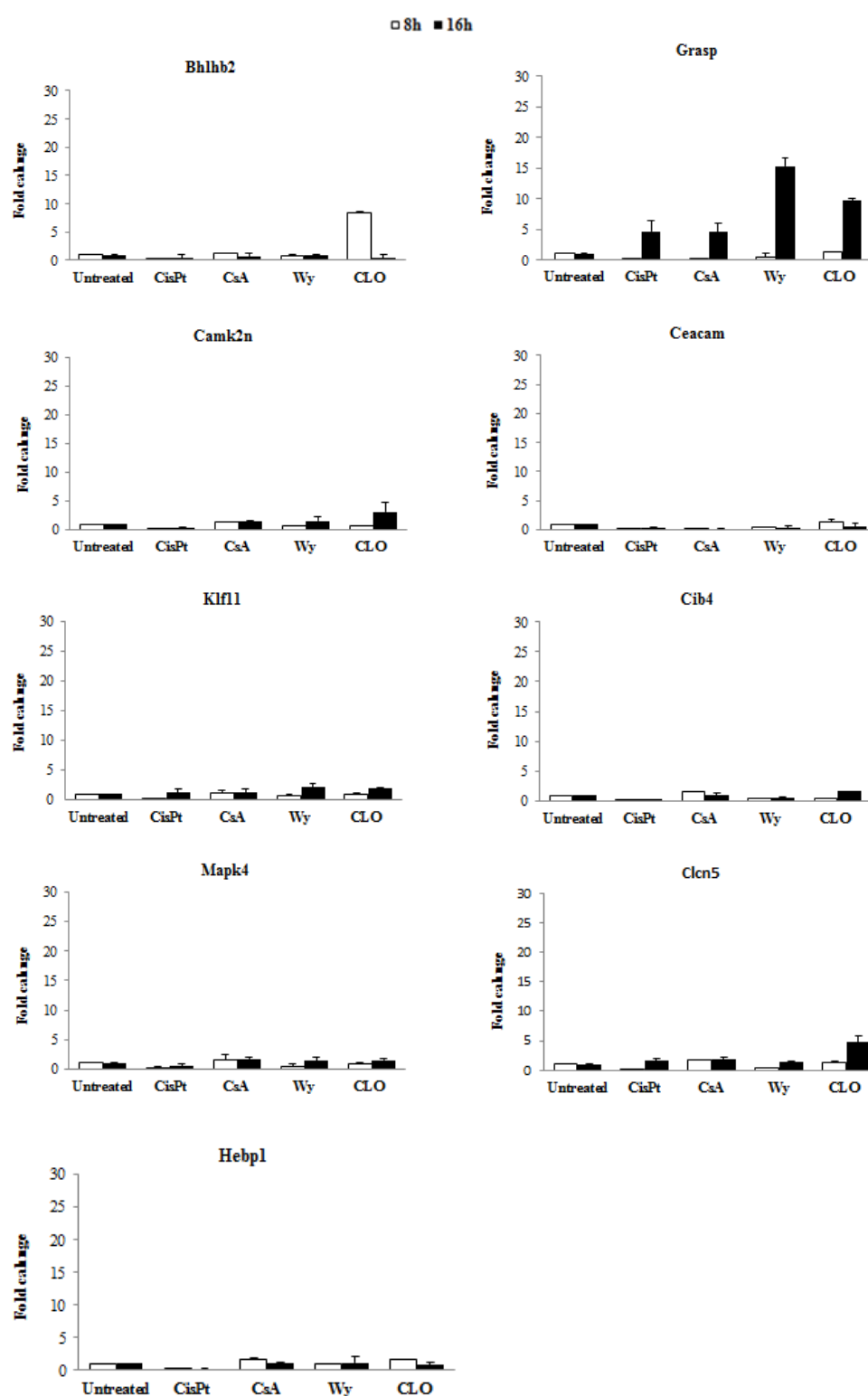


Fig. 4. Expression of biomarker genes for PPAR α ligands by qRT-PCR. Genes were tested on their expression with a qRT-PCR after 8 and 16 hrs exposure to CisPt 5 μ M, CsA 50 μ M, WY 250 μ M and CLO 400 μ M.

For the selected genes for metalloids we were able to select three genes: Krt8, Ddit3, and Atf3 (Fig. 5). These genes were selected because they showed the highest fold change after treatment, compared with the other genes. The gene Atf3 was the one that showed the highest fold change after exposure to sodium arsenite, after 16h of treatment. Krt8 and Ddit3 were induced by sodium arsenite, however the induction were less compared to Atf3. None of the genes showed induction when exposed to cisplatin. These genes were selected on genome wide transcription profiling based on their induction after treatment to sodium arsenite and PbAc, however the exposure to PbAc did not induce the genes, making us doubt the quality of the compound.

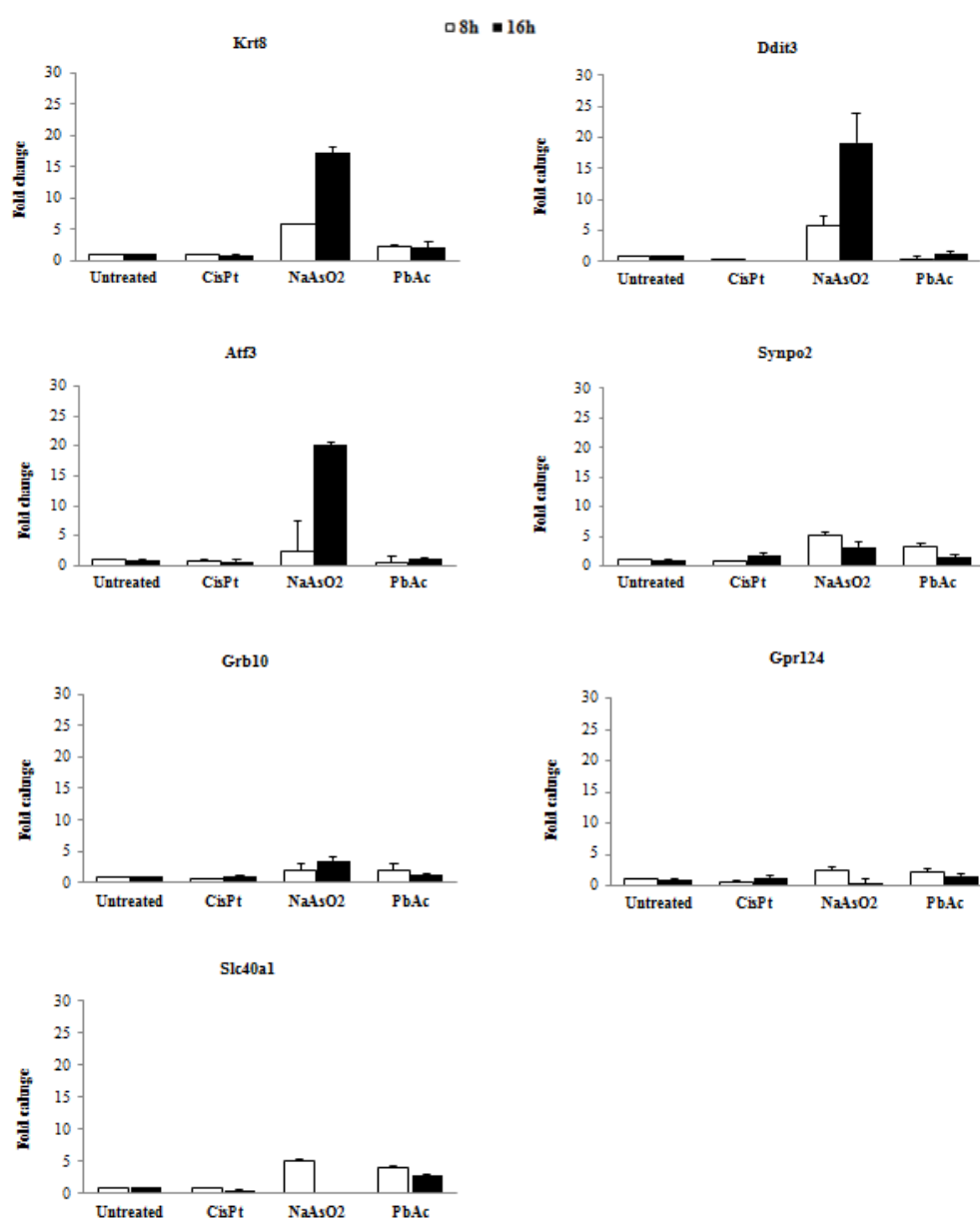


Fig. 5. Expression of possible biomarker genes for metalloids by qRT-PCR. Genes were tested on their expression with a qRT-PCR after 8 and 16 hrs exposure to CisPt 5 μ M, NaAsO₂ 5 μ M and PbAc 100 μ M. Krt8, Ddit3 and Atf3 were selected based on their induction to be biomarker genes for the metalloids.

Therefore, based on genome wide transcription profiling and on qRT-PCR we selected genes that were specifically activated after exposure to immune suppressor, PPAR α ligand and metalloids. The biomarker genes that were selected seem to be specific for non-genotoxic carcinogens, since they did not show any induction when exposed to cisplatin, a genotoxic compounds.

3.1.2. Development and selection of GFP-reporter cell lines

Next we generate the GFP-based reporter for each gene. Therefore, we used the BAC recE/T recombineering to develop the GFP-reporters, as previously described [1].

The biomarker genes that were selected for the immune suppressor, Armet, Derl3, Dnajc3 and H47, were previously generated. However, it was not possible to generate a GFP reporter for the gene H47. We developed GFP-based reporter cell line for the biomarker genes Bhlhb2, Grasp, Krt8, Ddit3 and Atf3.

The GFP-based reporter cell line was constructed in order to easily determine gene induction by the amount fluorescence emitted by the modified GFP fusion protein after compound treatment. First, electrocompetent BAC strains were transformed with the pRed/ET plasmid, which contains the RecE and RecT recombination enzymes. We generated PCR fragments encoding a GFP-ires-neomycin/kanamycin reporter cassette, using primers that contain an additional 50 nucleotides sequence homologous to the 3' sequence of the biomarker gene on the BAC (Fig. 6).

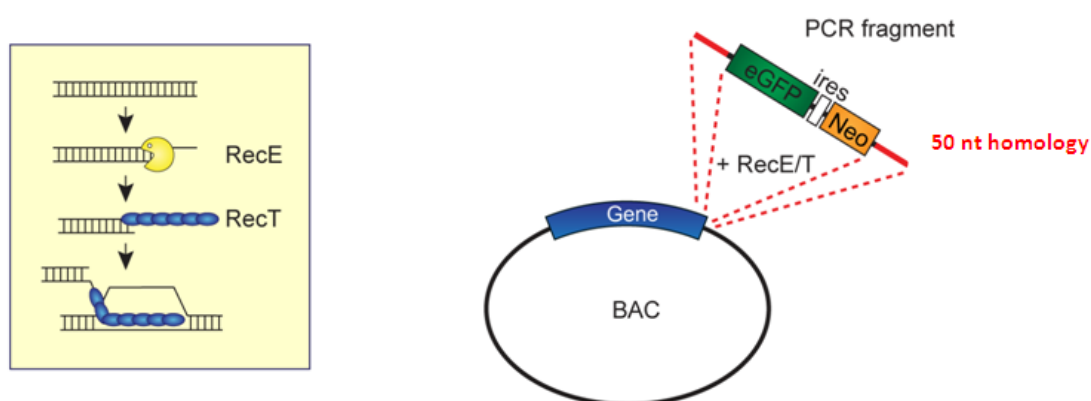


Fig. 6. Schematic representation of the RecE and RecT recombination enzymes and recombination of GFP-ires-Neo cassette into the BAC, adapted from Hendriks *et al.* [1].

The homologous sequences on both the 5'- and the 3'-ends of the PCR fragment allow RecE/T- mediated site-specific recombination of the GFP-ires-Neo selection cassette at the 3'-end of the biomarker gene on the BAC. Neomycin/kanamycin is a selection marker that allows the selection and IRES is an internal ribosome entry site, guaranteeing translation of the RNA.

Next, the BAC strains were transformed with the GFP-ires-Neo PCR fragment by electroporation. In order to establish the correct integration of the GFP cassette, we analysed each individual clones, by PCR and visualised by a gel electrophoresis (Fig. 7). The PCR products showed that for each gene there was at least one BAC successfully modified. The BACs containing the GFP-tagged biomarker genes were purified and analysed by sequencing.

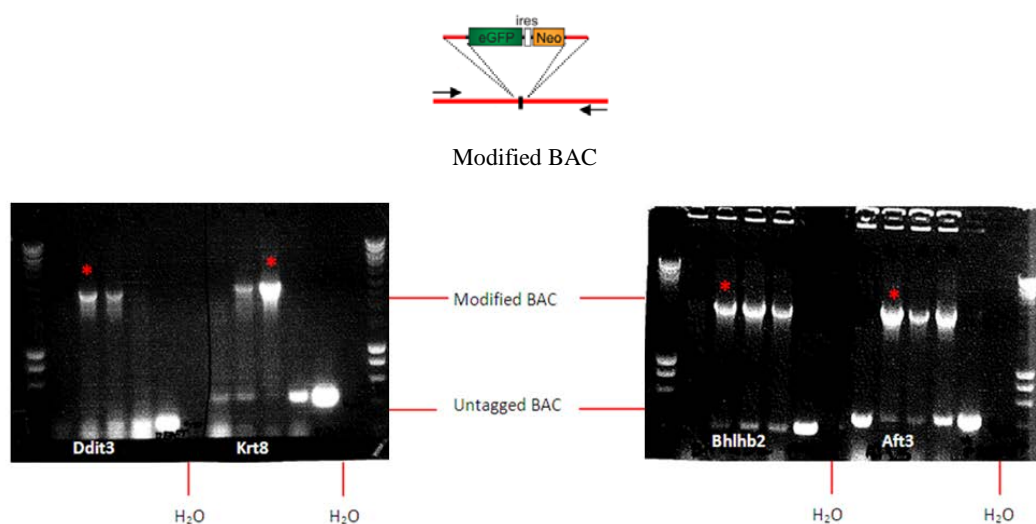


Fig. 7. Gel electrophoresis of PCR analysis of modified BAC-derived reporter genes. The modified BAC is 2.5 kb longer than the untagged BAC. The upper band represents the modified BAC and the lower band correspond a BAC without the GFP cassette. Clones with strong expression of the new modified construct were selected (red star) and purified.

Modified BACs with the genes of interest were transfected into the mES cells and cultured. mES were under selection of G418 for the presence and expression of the modified BAC. Approximately 15 day after, for each biomarker gene different colonies were selected.

Monoclonal mES cell lines were selected based on their level of induction of the GFP reporter after treatment to CLO or sodium arsenite, according to the non-genotoxic carcinogen that were selected, the GFP expression was determined by flow cytometry (Fig. 8).

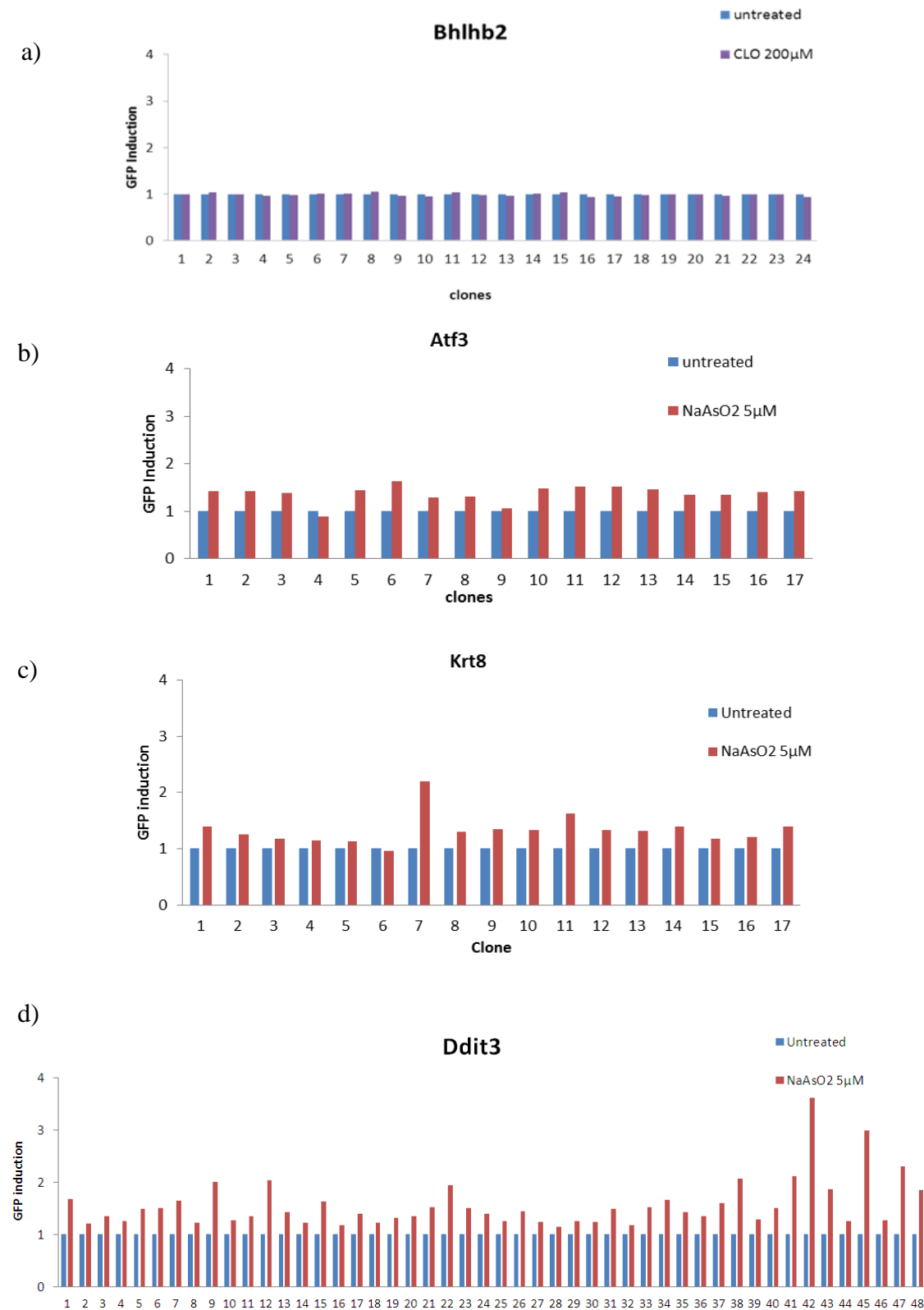


Fig. 8. Monoclonal mES cell line selection. a) Monoclonal selection for the biomarker gene *Bhlhb2* after treatment to CLO 200 μ M. b) Monoclonal selection for the biomarker gene *Atf3* after treatment to NaAsO₂ 5 μ M. c) Monoclonal selection for the biomarker gene *Krt8* after treatment to NaAsO₂ 5 μ M. d) Monoclonal selection for the biomarker gene *Ddit3* after treatment to NaAsO₂ 5 μ M.

We are able to develop GFP reporters for all biomarker genes that we select, however for the biomarker gene *Grasp* we were not able to introduce the GFP-cassette into the BAC strain.

The GFP reporters *Bhlhb2* and *Atf3* were successfully generated, nevertheless after treatment and flow cytometry analysis there were no clones showing significant GFP expression after treatment exposure (Fig. 8a and 8b).

For *Krt8*- and *Ddit3*-GFP we were able to select 6 clones each. For each clone the GFP induction was assessed by flow cytometry, and the amount of GFP was used to determine the clone which best represent the GFP-reporter (Fig. 9). Both cells cell lines were exposed to five increasing concentrations of sodium arsenite. All cells lines behave the same way.

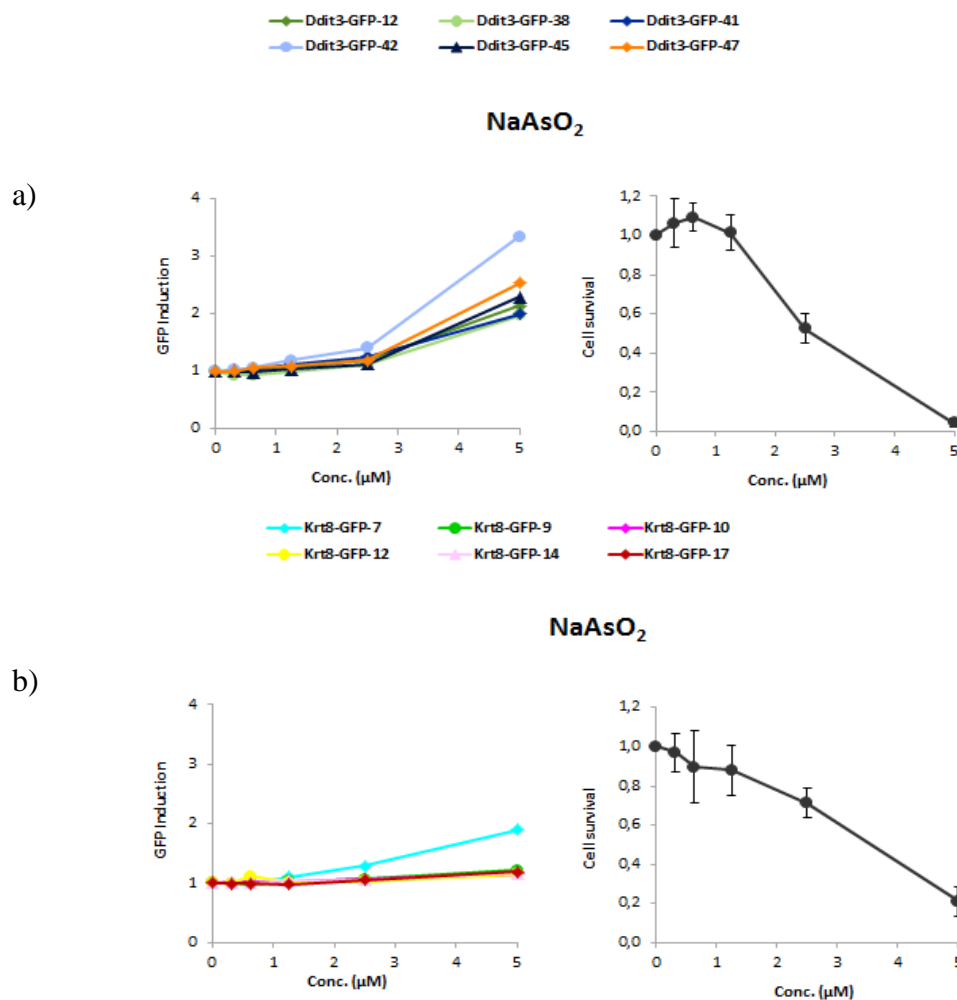


Fig. 9. Clone selection. a) Clone selection for *Krt8*-GFP, when exposed to five increasing concentration of NaAsO_2 . However we were not able to select clones, since none of them showed an increase of GFP induction. b) Clone selection for *Ddit3*-GFP, when exposed to five increasing concentration of NaAsO_2 . We select clone 42, since it was the one that showed the higher GFP induction. The cell survival was assessed by flow cytometry for each reporter, showing an increase of toxicity.

For the reporter Ddit3-GFP we select the clone 42, since was the one that showed the highest GFP induction after exposure (Fig. 9b). However, for the reporter Krt8-GFP we were not able to select one clone, because for all the clones the GFP induction after treatment was not significant (Fig. 9a). The cell survival was assessed, and with increasing concentrations there was an increase in toxicity, meaning that the clones for Krt8-GFP were not activated even though there was an apparent toxicity effect.

The biomarker genes Bhlhb2, Atf3 and Krt8 were selected based on their high expression by qRT-PCR (Fig. 4 and Fig. 5), although we were able to developed GFP-reporters for all the biomarker genes, the GFP expression was not relevant for these reporter. We can point the cause of the lack of induction to the stability of the GFP protein. If the protein is not stable and degrades, we could not measure the GFP expression.

3.1.3. GFP-based reporters: sensitivity and specificity

We were able to successfully develop a reporter for non-genotoxic carcinogen, which was selected after treatment to sodium arsenite, together with GFP reporters that were previously develop for immune suppressor, Armet-, Dnajc3- and Derl3-GFP we have a set of reporter. Therefore, the next step was to assess the sensitivity and specificity of the mES of Armet-, Dnajc3-, Derl3- and Ddit3-GFP.

It has previously been described that Armet is mediated by ERSE-II response element, Dnajc3 is mediated by ATF6, Derl3 is a target for IRE1/XBP1 and Ddit3 encodes for CHOP [35]. Thus, meaning that all the reporters are related with the unfolded protein response (UPR).

To verify sensitivity and specificity we start to expose the reporters to five increasing concentrations of sodium arsenite, cyclosporin A and WY, and then measure the GFP expression by flow cytometry (Fig. 10).

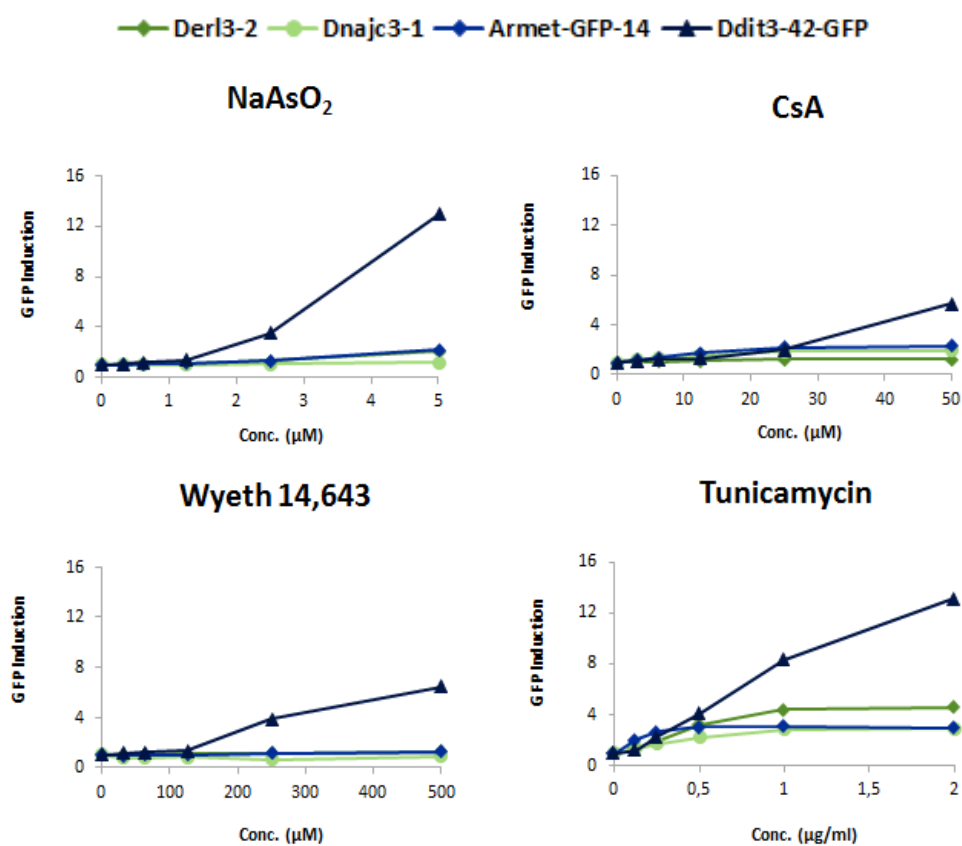


Fig. 10. The mES reporter cell lines respond preferentially to compounds that induce protein damage. GFP reporter cells were exposed to NaAsO₂, CsA, Wyeth 14.643 and tunicamycin. 24h after exposure the GFP expression was determined by flow cytometry

It was previously described that immune suppressor cyclosporin A and metalloid sodium arsenite are related with the UPR [36,37], and we confirm after exposure to these compounds, that these GFP-reporters were induced either by cyclosporin A and sodium arsenite (Fig. 10). The reporters that were developed for the immune suppressor were induced just when exposed to cyclosporin A. Ddit3-GFP was induced after treatment to sodium arsenite, cyclosporin A and Wyeth-14.643 (Fig. 10).

To confirm that the mode of action of these reporters was the UPR, we exposed the GFP-reporters to tunicamycin, which is a compound that induces the UPR [38]. After exposure, all the reporters were induced, although Ddit3-GFP showed the higher GFP expression. We test the GFP-reporters with genotoxic compounds that induce DNA damage and oxidative stressor to check if they are specific only of non-genotoxic carcinogens (Fig. 11).

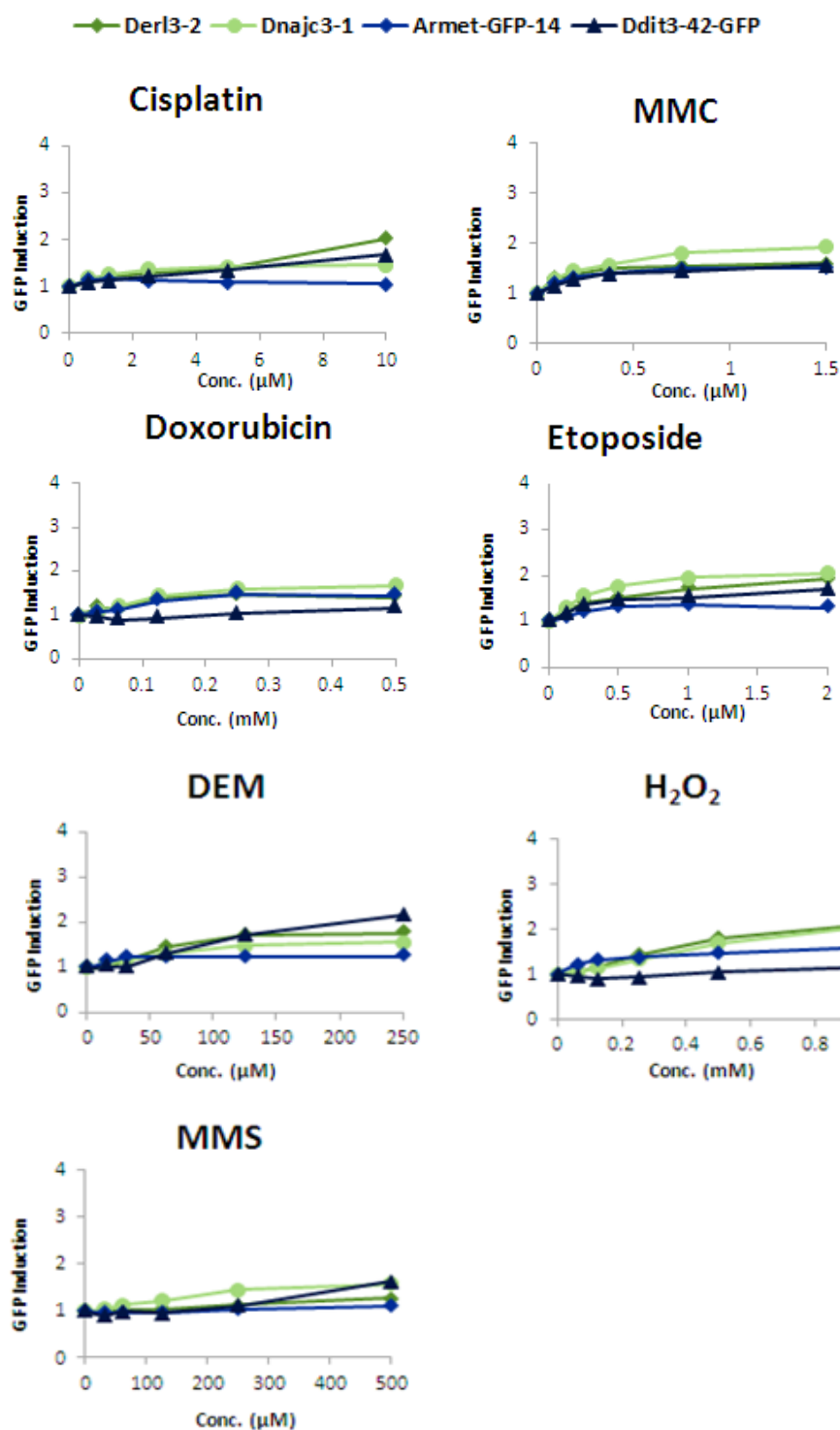


Fig. 11. Check the sensitivity and specificity of the GFP-reporter. The GFP reporter cells were exposed 24h to DNA-damaging agents: Cisplatin; MMC; doxorubicin; and etoposide, and to oxidative stress-inducing agents: DEM, H₂O₂ and MMS. The GFP induction was assessed 24h after treatment by flow cytometry.

When the GFP-reporters cells lines developed for the UPR were exposed to genotoxic compounds, they did not show any induction, or the induction was not significant (Fig. 11), showing that the GFP-reporters that we developed are specific for non-genotoxic carcinogens and aren't induced when exposed to genotoxic compounds. We assessed the cell survival for all the tested compounds, although the compounds did not induce the reporters, with increasing concentrations there was an increase in toxicity, and we observed that the lack of induction of GFP-reporter when exposed to genotoxic compounds was not due to the condition of the compound, but the lack of induction by compounds (supplemental figure 1).

Therefore, we were able to developed reporters that were induced by the UPR, and there are specific for non-genotoxic carcinogens, especial to CsA and NaAsO₂.

3.2. Validation of the GFP-reporters for non-genotoxic carcinogens

Thus, the next step was to establish the sensitivity and specificity of the Armet-, Dnajc3-, Derl3- and Ddit3-GFP reporters, together with Sdc1, Eng, Rtkn, Btg2 and Blvrb-GFP reporters.

The Sdc1-, Eng-, Rtkn-, Btg2- and Blvrb-GFP are reporters that were previously developed to detect carcinogenic properties of genotoxic carcinogens compounds (table 3). They can distinguish between DNA damage, cellular stress and oxidative stress. The GFP-reporters Sdc1, Eng and Rtkn are reporters that are specifically induced when mES cells are exposed to genotoxic compounds. The Btg2-GFP is a p53-dependent, which is responsive to DNA-damaging agents and pro-oxidants [21]. And the Blvrb-GFP is induced after exposure to chemicals that result in increased levels of cellular oxidative stress.

Table 3

Various GFP reporters and the biological effects.

	Biological damage	Biomarker gene
NGTXC	UPR	Armet
		Dnajc3
		Derl3
		Ddit3
		Sdc1
GTXC	DNA damage	Eng
		Rtkn
	Cellular stress	Btg2
	Oxidative stress	Blvrb

These nine GFP-reporters were exposed to a broad range of carcinogenic and noncarcinogenic compounds, as suggested by the ECVAM [33]. Those compounds are divided in three classes. The ECVAM Class 1 includes the compounds that are *in vivo* genotoxins carcinogens with a mutagenic mode of action, should score positive in an *in vitro* genotoxicity assay. The ECVAM class 2 includes non-genotoxic carcinogens or noncarcinogens with a nonmutagenic mode of action, should be negative in *in vitro* genotoxicity tests. And the ECVAM class 3 includes noncarcinogens with a nonmutagenic mode of action. Negative in *in vivo* genotoxicity studies, but nonetheless they have been reported positive in some *in vitro* genotoxicity tests.

In total, the GFP-reporters were exposed to 53 different compounds, mainly ECVAM-suggested genotoxins, pro-oxidants, and nongenotoxins (table 4 and Fig. 12).

Table 4

Tested compounds for the validation of the GFP-reporters. 35 compounds have been proposed by the ECVAM [34] and 18 compounds are additional to the ECVAM.

Compound	Highest conc. for mES cell exposure (μM)
ECVAM class 1	
Cisplatin	10
Methyl methosulfate	500
Cadmium chloride	20
p-chloroaniline	1000
Sodium arsenite	5
Taxol	1
Etoposide	2
ECVAM class 2	
n-butyl chloride	10000
Phenformin HCl	1000
(2-Chloroethyl) trimethy NH ₄ Cl	10000
Dicyclohexyl thiourea	10000
Cyclohexanone	10000
Erythromycin stearate	2500
Fluometron	1000
D-Limonene	1000
Amitrole	10000
Tert-butanol	1000
Diethanolamine	10000
Hexachloroethane	1000
Methy carbamate	10000
Pyridine	10000
tris (2-ethylhexyl)phosphate	10000
2-chloroethanol	10000
ECVAM class 3	
Anthranilic acid	10000
Resorcinol	1000
Sulfizoxazole	1000
Ethionamide	10000
Benzyl alcohol	10000
Sodium saccharin	10000
Nitrophenol	1000
2,3-dichlorophenol	1000
Eugenol	1000
Ethyl acrylate	1000
Isobutyraldehyde	1000
Propyl gallate	1000
Additional genotoxins	
Doxorubicin	0.5
Mitomycin C	1.5
Diethyl maleate	250
Hydrogen peroxide	1000
Copper sulfate	400
Potassium bromate	2500
4-Nitroquinelone-1-oxide	2
4 Hydroxy-2-nonenal	40
Cytarabine	50
Camptothecin	0.3
Hydroxyurea	1000
1-Nitropyrene	100
Non-genotoxic carcinogens	
Cyclosporin A	50
Tacrolimus	25
Wyeth-14643	500
Clofibrate	100
Lead Acetate	100
Tunicamycin	2

To study the GFP expression of the reporters, we develop a software, the ToxPolt that is able to create a heatmap that plots the GFP expression against 50% toxicity given by the compound. After compound exposure the ToxPlot cluster the GFP-reporters based on their specificity, being possible to discriminate between compounds that give DNA damage, oxidative stress and protein damage.

All ECVAM class 1 compounds scored positive for Sdc1-, Eng-, Rtkn-, Btg2- and BlvrB-GFP reporters cell lines, except p-chloroaniline. Although this compound is an *in vivo* carcinogen, it was also not identified as genotoxin in other *in vitro* genotoxicity assays [1]. The reporters for UPR scored negative for all the ECVAM class 1, indicating that these GFP-reporters are specific only for non-genotoxic carcinogens, therefore are not induced by these class of compounds (Fig. 12).

All tested ECVAM Class 2 compounds failed to induce the GFP reporters for the genotoxic carcinogens. However, the compounds n-butyl chloride, phenformin HCL, 2-chloroethyl trimethyl NH₄Cl, dicyclohexyl thiourea, erythromycin stearate and tris 2-ethylhexylphosphate induced the reporter Ddit3-GFP. The ECVAM Class 2 included non-genotoxic compounds, suggesting that the activation of Ddit3-GFP is associated with the toxic properties of these compounds, with the induction of protein damage, and fits with an assumed non-mutagenic mode of action (Fig. 12).

The compounds of ECVAM class 3 failed to induce all the GFP-reporters. However, the compounds sulfoxazole, benzyl alcohol, eugenol, ethyl acrylate and propyl gallate induced the reporters for GTXC suggesting that these compounds contain *in vitro* genotoxic properties even though exposure to these compounds has not been found to cause cancer [1], while the compounds resorcinol, p-nitrophenol and dichlorophenol induced the reporters for the UPR, that fits with an assumed non-mutagenic mode of action (Fig. 12).

An additional set of 18 non-ECVAM suggested compounds were tested to identify the specificity of the GFP-reporters, and were all correctly identified as genotoxic, oxidative stress or protein damage. Nitropyrene was one of the additional compounds that induced both non-genotoxic carcinogens and genotoxic carcinogens GFP-based reporters. This compound induces DNA damage, give oxidative stress and protein damage, and is associated with carcinogenicity [38] (Fig. 12).

Together, these results show that the reporters for genotoxic carcinogens (Sdc1-, Eng-, Rtkn-, Btg2- and BlvrB-GFP) and for non-genotoxic carcinogens (Armet-, Dnajc3-, Derl3- and Ddit3-GFP) can provide a sensitive and selective assay to establish potential toxic activities of compounds, and provide insight in the primary toxic properties of compounds.

To show the capacity of the GFP-reporters, we selected just the reporters that gave the stronger GFP expression after treatment for each group. For the UPR we selected the reporter Ddit3-GFP, for DNA damage we selected Rtn-GFP, and for cellular stress and oxidative stress we selected Btg2 and Blvr-GFP, respectively. With these four GFP reporters we showed that they were able to distinguish between compounds that induce DNA damage, protein damage and give oxidative stress. We selected the compounds that were score positive for each group of reporters, and we create a heatmap where it's visible that the reporter for UPR is strongly induce by cyclosporin A and tunicamycin. The Blvr-GFP is strongly induced by MMS and DEM and Btg2 and Rtn were both induced by cisplatin and mitomycin C (Fig. 13).

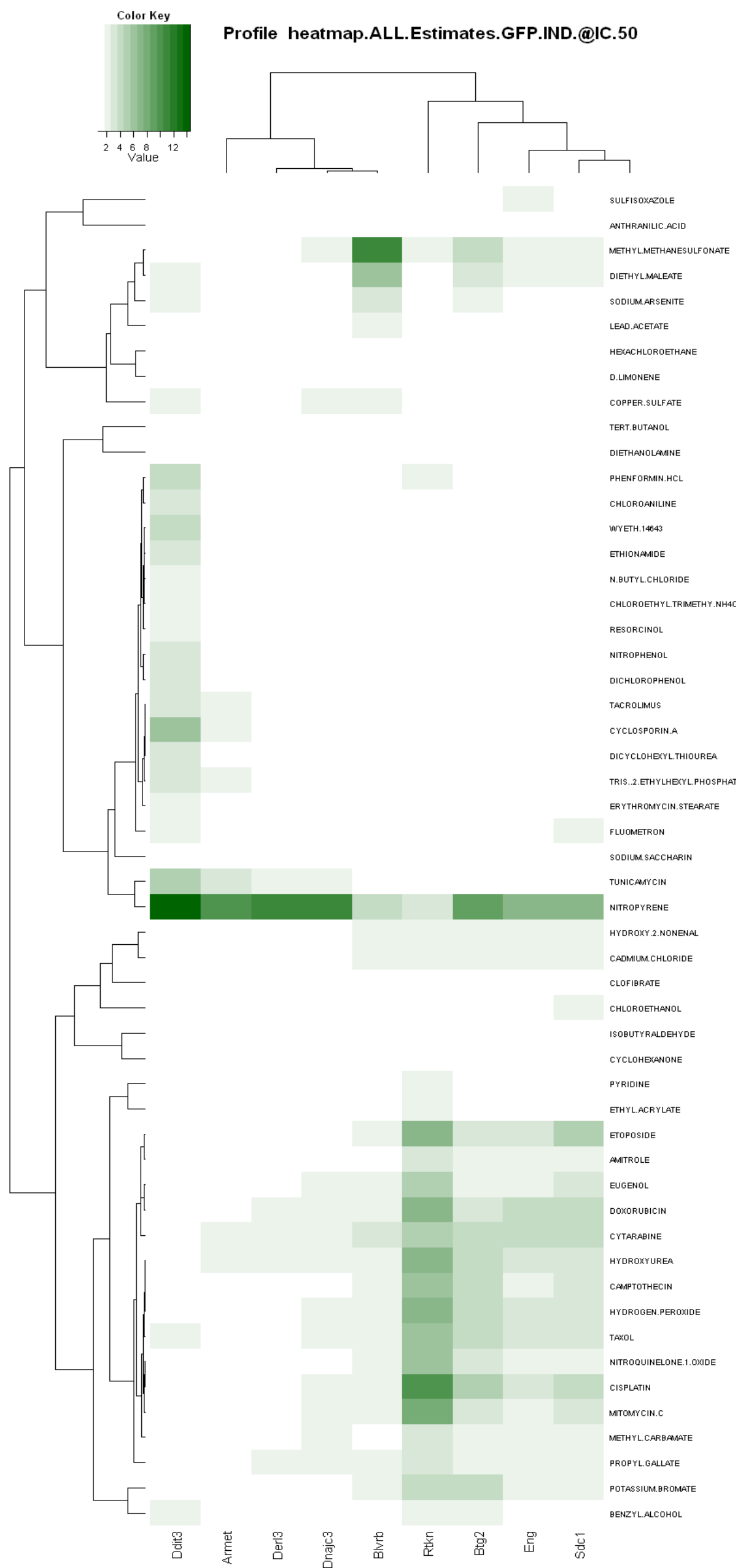


Fig. 12. Validation for the reporters, tested with 53 compounds suggested by ECVAM. The GFP-reporters cell lines were exposed to different compounds for 24h and the induction in total GFP was assessed by flow cytometry. The ToxPlot create the heatmap was made based on GFP expression at the 50% toxicity

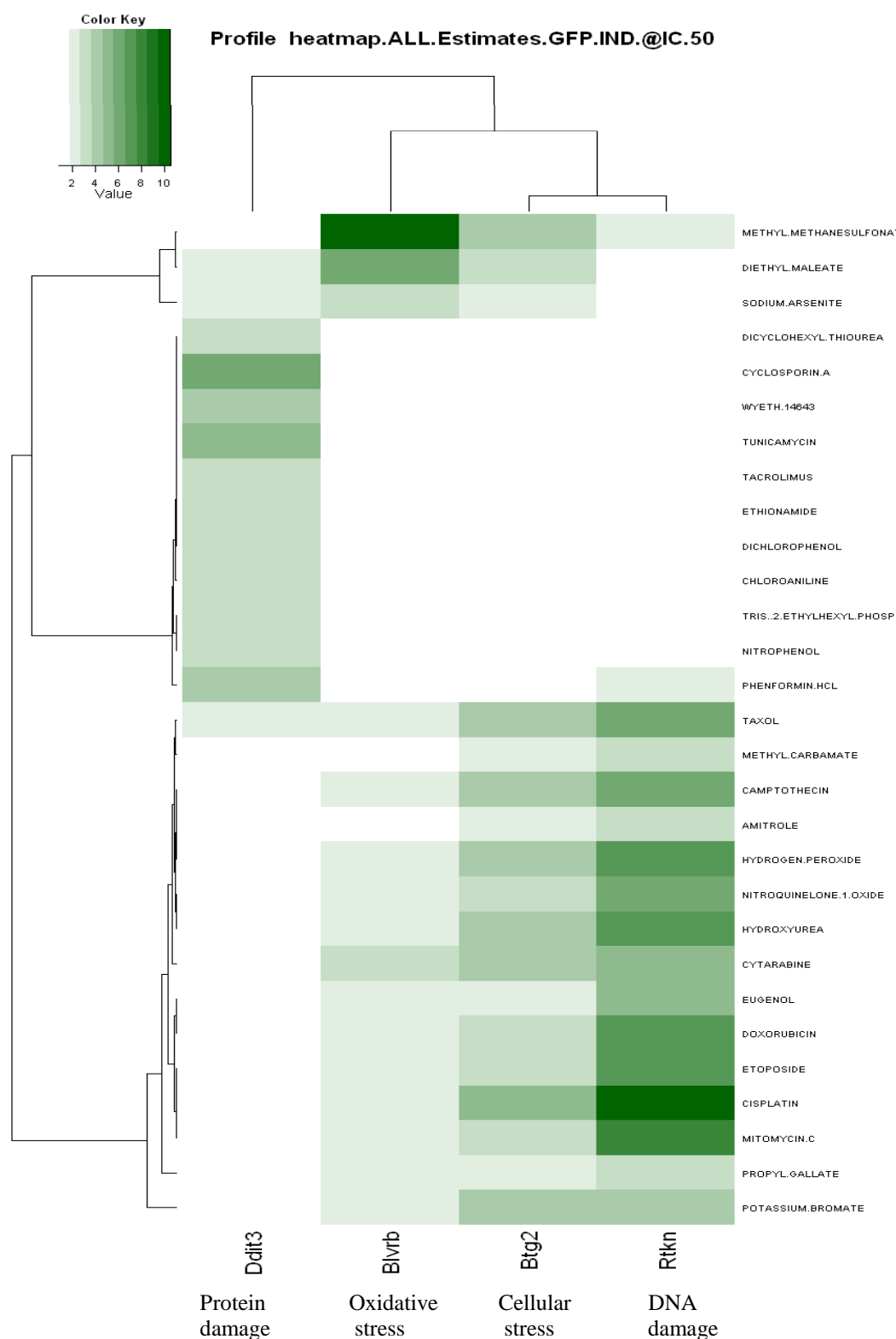


Fig. 13. ToxPlot for Ddit3-GFP, Blvrb-GFP, Btg2-GFP and Rtkn-GFP. mES reporter cells respond preferentially to protein damage, pro-oxidants or genotoxic compounds, respectively. Selection of compounds that induce our reporters, the GFP expression was assessed 24h after treatment by flow cytometry. The heatmap plots the GFP induction at 50% toxicity.

We were able to develop and validate GFP-reporters cell lines for non-genotoxic carcinogens and genotoxic carcinogens. The GFP reporters Armet, Dnajc3, Derl3 and Ddit3 are induced by compounds that give protein damage, and did not show any induction when exposed to compounds that give DNA damage or pro-oxidants, although the expression of Ddit3-GFP was higher compared with the other reporters for the UPR. These reporters are specific for non-genotoxic carcinogens that induce protein damage. The reporters Sdc1-, Eng-, Rtkn-, Btg2- and Blvrb-GFP are specific for genotoxic carcinogens, and are induced either by DNA damage or oxidative stress.

3.3. Comet assay

In order to correlate the GFP-reporter activation with a biological endpoint we use an *in vitro* assay to establish that activation. We selected the comet assay, because under alkaline conditions (pH >13), the assay can detect single and double-stranded breaks, alkaline labile sites and excision repair processes in individual cells [22,39]. Following the single-cell electrophoresis, the lengths of the comets (DNA tails) depended on the treatment, concentration and exposure time, in which longer tails indicate a higher DNA damage.

Nevertheless, there are no available protocols for comet assay using the mES, therefore we had to optimize the assay, and once the protocol was working we could test the chemicals and establish the reporter activation.

To correlate the reporter activation with a biological endpoint, we had to expose the mES used in the comet assay with the same chemicals used for the validation of the GFP-reporters, thus we test 23 chemicals (Fig. 14) that belong to table 4. The compounds used in the comet assay were selected based on the induction of a reporter, or in cases that did not lead to reporter activation the chemical showed high levels of toxicity. For all the chemicals, we tested different concentrations and time points (supplemental table 1) and for each compound we plot the higher tail moment (Fig. 14).

We consider a negative result (no tailing) when the value for tail moment is lower than 0.0912 (five time higher that the SD in untreated cells), and a positive result when the tail moment is above that value.

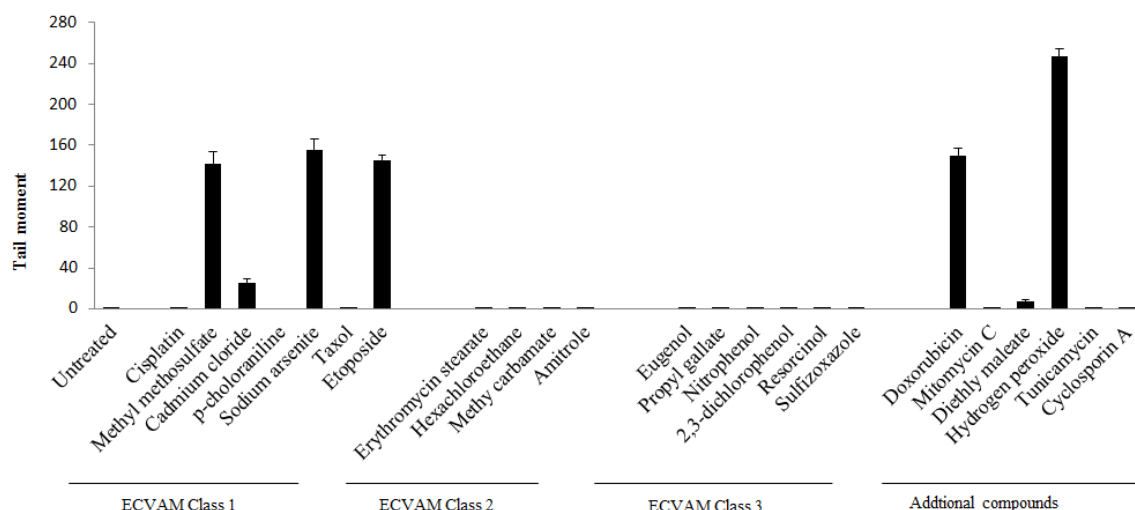


Fig. 14. Alkaline comet assay. We test 23 compounds divided in 4 classes: the ECVAM class 1, 2 and 3 and additional compounds. For each compound we plotted only the higher tail moment (mean \pm SD of at least 50 cells).

The chemicals belonging to the ECVAM class 1 induced DNA breaks, which we can be correlated with the reporter's activation, since they have a mutagenic mode of action [33]. Yet, cisplatin and taxol are both negative in the comet assay, and are chemicals that strongly induce the GFP-reporters for DNA damage. However it has been described that cisplatin reacts with DNA, leading to the formation of inter-strand and intra-strand cross-links and decrease the DNA migration, although induces the GFP-reporters, in comet assay lead to a reduction of the tail [40,41,42].

The chemical p-chloroaniline induced the GFP-reporter Ddit3, a reporter for protein damage (Fig. 12 and Fig. 13), however, in the comet assay this chemical was scored negative, leading us to believe that the comet assay cannot detect protein damage.

Chemicals from the ECVAM class 2 and 3 did not induced DNA damage that could be detected by the comet assay. These classes of chemicals include non-genotoxic carcinogens and noncarcinogens [33] that cannot be measurable by the comet assay. However, using the combination of GFP-reporters we were able to detect DNA damage caused by these chemicals (Fig. 13).

We tested six additional compounds (Fig. 14), and were selected because they induce our GFP-reporter. These chemicals were scored positive in the comet assay, however mitomycin C, tunicamycin and cyclosporin A failed to induce a comet tail.

Mitomycin C is well known cross-linking agents and induces DNA breaks [9,43], however reduces DNA migration, that is why is scored negative in the comet assay [40,41,42].

Tunicamycin and cyclosporin A are both non-genotoxic carcinogens that lead to the activation of the reporter Ddit3-GFP, specific for protein damage, however these chemicals couldn't induce tail in the comet assay, causing us to question the sensitivity of the assay. Therefore, we can state that the GFP-reporters are much more sensitive and specific to test the genotoxic properties of a compound.

We performed the comet assay under neutral conditions (Fig. 15), and although the neutral and the alkaline comet assays detect both single- and double-strand breaks and it is impossible to distinguish between them, the results in the alkaline comet have proved to be much better.

On the neutral comet the negative control has a big background that makes it difficult to analyze and compare between chemicals, however we reach the same conclusions.

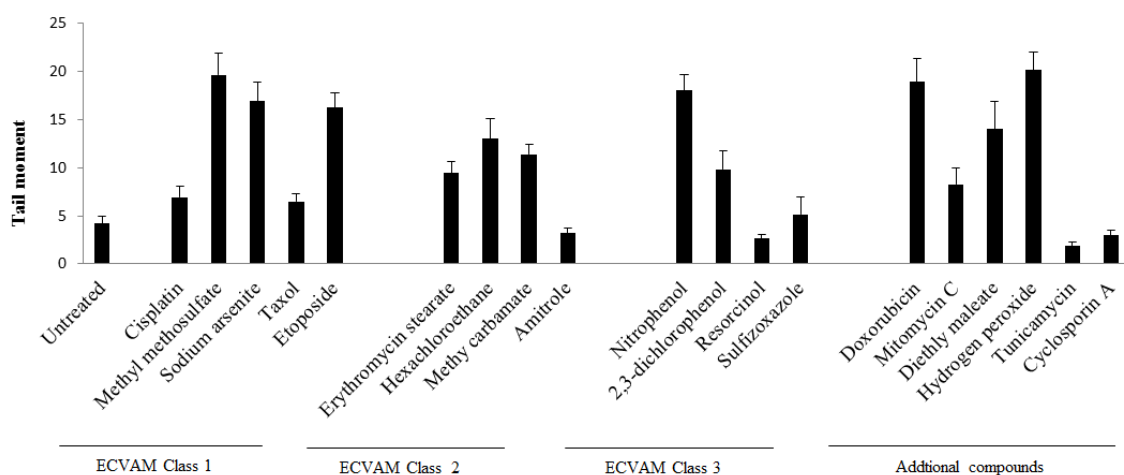


Fig. 15. Neutral comet assay. We test 19 compounds divided in 4 classes: the ECVAM class 1, 2 and 3 and additional compounds. Only the higher tail moment for each compound was plotted (mean \pm SD of at least 50 cells).

The ECVAM class 1 induces DNA breaks, except cisplatin and taxol. ECVAM class 2 and 3, in the neutral comet induced a comet tail, but in some cases are not

relevant. And in the additional compound, mitomycin A, tunicamycin and cyclosporin A fail to induce a comet tail.

Therefore, using the combination of different GFP-reporters we can predict the genotoxic properties of compounds. Using the traditional assay, like comet assay, to measure the genotoxic properties, we cannot distinguish between chemicals that give DNA damage, oxidative stressor or protein damage.

Is much wiser to use an assay that can detect possible carcinogenic properties of non-genotoxic compounds, once traditional assays are not able to do it, so using the reporters that we developed for the UPR we can detect these properties, that otherwise was not possible to do using an *in vitro* assay.

Chapter 4

Discussion

The aim of our research was to develop an *in vitro* assay, a GFP-based report assay, for the identification of possible carcinogenic properties of non-genotoxic compounds, and build up hypotheses about its molecular mode of action. Here, we described a set of genes, Armet, Dnajc3, Derl3, and Ddit3, which consists of different GFP fluorescence mES reporter cell lines that are preferentially responsive to protein damage.

Although have not yet been identified marker for non-genotoxic carcinogens, with this project we have selected biomarker genes for a subset of non-genotoxic carcinognes. These markers show an involvement in the unfolded protein response (UPR), when mES were exposed to certain non-genotoxic carcinogens, namely the immune suppressor's cyclosporin A and tacrolimus, and metalloid sodium arsenite (Fig. 10). Although still much disputed the UPR has been associated with carcinogenesis [44]. Some research even shows that the UPR is required for carcinogenesis [45].

Cells are able to activate a series of complementary adaptive mechanisms to cope with the damaged and unfolded proteins, which is known as the unfolded protein response (UPR) (supplemental fig. 2). Unfolded protein signalling originates from the endoplasmic reticulum (ER) or cytoplasm [46]. ER stress triggers the UPR as a mechanism to limit cellular damage, to initiate repair of misfolded proteins, to dispose of irreparable proteins, and to initiate programmed cell death in the context of extreme ER stress.

UPR is a complex network of signalling events that target multiple cellular responses and is associated with the activation of three major stress sensors: inositol-requiring protein 1 (IRE1) (both α and β isoforms), transcription factor 6 (ATF6) (both

α and β isoforms) and protein kinase RNA-like ER kinase (PERK) [46,47,48]. In the unstressed ER, these sensors are associated with the chaperone, glucose regulated protein 78 kDa (GRP78/BiP) [46]. Upon unfolded/misfolded proteins accumulate in the ER, GRP78 is released from the sensor proteins, enabling activation of their three pathways [7,47]. PERK activation leads to phosphorylation of the α subunit of the eukaryotic translation initiator factor 2 α (eIF2 α) to attenuate general protein synthesis. The phosphorylated eIF2 α allows the translation of ATF4 mRNA, which encodes a transcription factor controlling the transcription of genes involved in autophagy, apoptosis, amino acid metabolism and antioxidant responses [48]. Upon dissociation of GRP78, IRE1 α dimerization, followed by autotransphosphorylation, triggers its RNase activity, which processes the mRNA encoding spliced X-box binding protein 1 (XBP1u) to produce an active transcription factor, spliced XBP1 (XBP1s). The XBP1s controls the transcription of genes encoding proteins involved in protein folding, ER-associated degradation (ERAD), protein quality control and phospholipid synthesis [47,48]. ATF6 is localized at the ER in unstressed cells, although, after dissociation of GRP78, ATF6 is transported to the Golgi apparatus through interaction with the coat protein II (COPII) complex, where it is cleaved by site 1 and site 2 proteases (S1P and S2P), releasing its cytosolic domain fragment (ATF6f) [47,48]. ATF6f controls the upregulation of genes encoding ERAD components and also XBP1 [48].

The promoter of most of the UPR-target genes contain the ERSE sequence leading to their activation [49]. The protein chaperones play an important role in the UPR. They are involved in the holding and folding of proteins [50], they bind to unfolded sequences and show preference for hydrophobic parts of the protein. They do this to prevent premature associations with other proteins, during heat shock when proteins tend to unfold, and to stabilize unstable proteins [51]. The chaperones that are activated in the UPR are members of the heat shock proteins. The heat shock proteins have been identified as factors that play a role in one or more of the steps leading to carcinogenesis [51]. The binding of certain heat shock proteins is required for the stability of proteins involved in pathways leading to carcinogenesis, such as the factors of extracellular-regulated-signal-kinase (ERK) pathway and the pro-survival PI3K pathway and heat shock proteins also help by evading fast programmed cell death [51]. Certain heat shock proteins also facilitate the emergence of polymorphisms and mutations by stabilizing mutated proteins [9, 15].

As mentioned previously *Armet*, *Danjc3*, *Derl3* and *Ddit3* are involved in the UPR. *Armet* (arginine-rich mutated in early stage tumors) it has been proven to be ER-stress responsive gene [52]. *Armet* contains intra-molecular disulfide bonds and is a

soluble protein resident in the ER (27-aster). Armet induction requires either ATF6 or XBP1 [52]. The UPR of *Armet* is mediated by ERSE-II response element, suggesting that Armet is involved in protein-quality control [53, 54]. There are studies showing that the P58^{IPK}, a protein homologs to Dnajc3 (Interferon-induced, double-stranded RNA-activated protein kinase inhibitor) is implicated in the UPR [55]. The induction of P58^{IPK} has been mediated by ATF6 and contributes to the down-regulation of the PERK/eIF2 α /ATF4 response pathway [56,57]. Derl3 (Der1-like domain family, member 3) is up-regulated by UPR, and plays as role in the ERAD for misfolded glycoproteins and that it is a target for IRE1/XBP1[58]. Ddit3 (DNA-Damage-Inducible Transcript 3) is induced by UPR, and encodes for the C/EBP homologous protein (CHOP) [46]. CHOP was the first identified protein that mediates ER stress-induced apoptosis [48, 59].

By, flow cytometer we showed that Armet, Dnajc3 and Derl3 were preferentially induced by immune suppressors cyclosporin A and FK506, and Ddit3 by the metalloid sodium arsenite (Fig. 10). However, when we exposed the GFP-reporters to DNA damage or oxidative stressor the GFP expression was either weak or not significant (Fig. 11).

Cyclosporin A and Fk506 are immunosuppressive agents used to avoid transplant rejection and to treat autoimmune diseases. The mode of action of their immune suppression is based on their interference with T-cell activation and T-cell growth by inhibition of transcription of early lymphokines [29]. Du *et al.* [29] report that administration of cyclosporin A in a reporter mouse induced the UPR *in vivo*. The GFP-based reporter Armet, Dnajc3 and Derl3, showed to be induced by cyclosporin A (Fig. 10), therefore, our results indicate that both cyclosporin A and FK506 lead to the activation of the UPR. The mode of action of metalloids, it's still unclear. However, sodium arsenite is able to activate the promoters of a great number of genes that are involved in differentiation, proliferation and apoptosis [60], and can induces protein misfolding, as well as protein aggregation [48].

Thus, using the reporter Ddit3-GFP we settle that sodium arsenite activate the UPR, however Ddit3 was also induced by cyclosporin A. This chemical induces CHOP [29] confirm what we saw on Fig.10, that Ddit3 was induced either by cyclosporin A and sodium arsenite. On the other hand, when mES GFP reporter were exposed to tunicamycin, a compound which is commonly used as positive control in UPR, since it interferes with the glycoprotein synthesis causing ER-stress [46], they showed GFP induction (Fig. 10). Together with the data from the expose to nitropyrene (Fig. 12), that gives protein damage and is associated with carcinogenicity [37], and induces the four GFP-reports. Therefore taken together, these suggest that the reporters that we develop

are biomarker genes are good candidates for non-genotoxic carcinogens, involved in the UPR, and are good biomarker genes to detect protein damage. However, the GFP expression of Ddit3-GFP when exposed to all compounds was comparatively higher than Armet, Dnajc3 and Derl3, thus we affirm that Ddit-GFP is the best candidate to detect protein damage. Although many players of the UPR, such as the protein chaperones have previously been associated with carcinogenesis [1,7,11], this makes the correlation between UPR activation and carcinogenesis an obvious one.

All of the already developed cell lines showed induction of GFP after treatment, from which can be concluded that the fusion protein is synthesized in all cell lines. However, the amount of GFP induction of mRNA is not in accordance with the amount of fusion protein synthesized, causing the GFP induction to be just a part of the total mRNA induction. This difference could be caused by a number of effects, which all need to be investigated before the further validation of the cell lines continues. Therefore, before the reason for this difference is investigated it is essential to first determine whether the activation of the reporter is representative for the endogenous gene activation by means of qRT-PCR and with a Western blot on protein level. When both endogenous and fusion gene activation are similar the difference in mRNA and protein level can be further investigated. The first possibility for the difference may merely be time, as mRNA is isolated after 8 and 16h and the GFP induction is assessed after 24h. However, protein synthesis takes longer than mRNA transcription and therefore the optimum of protein synthesis may lie respectively later or earlier than 24 h. Thus cells should be treated for different time periods to look for the optimal GFP induction. It could also be that the cell produces more mRNA than is actually necessary for protein synthesis. But, this is thought of as highly unlikely as under selection pressure a cell looks for the optimal use of energy and production of extra mRNA would be a waste of energy. Another possibility to explain the difference in mRNA and protein level is that the fusion protein is not stable or the protein is usually fast degraded after synthesis. It is therefore essential to assess the kinetics of the fusion protein. Kinetics can be assessed by bleaching of the GFP reporter and then determine how quickly new proteins are synthesized. One could also block all protein degradation in order to see whether the GFP-induction rises. However one cannot specifically block the degradation of the fusion protein and thus other cell mechanisms might influence the outcome.

It is also advisable to assess the location of the fusion protein under the microscope to assess the exact location of the proteins in the cell and whether this is in

accordance with the endogenous protein. In conclusion much has to still be investigated before one can continue with the development of these cell lines.

Even though the development of a validated GFP based reporter cell lines for the identification of possible carcinogenic responses of NGTX carcinogens is not finished it is important to discuss the representativeness of the mES cell lines for the induction of cancer in humans. Even though these reporters are based on mES cells, they are still be very representative for the human model, because the strategy to deal with ER-stress by the UPR is well conserved from yeast to humans [10]. Therefore, it adds to the probability that these mES reporter cell line when finished have the same predictive value as human reporter cells lines and would therefore be very applicable for the identification of chemicals that have a possible carcinogenic effect in humans. Although it is likely that this *in vitro* assay is representative for *in vivo* risk assessment this does has to be validated. Stem cells are untransformed non-cancerous cell lines that are proficient in the main DNA damage signaling and cell-cycle control systems and are genetically stable, on the contrary most commonly used cell lines in *in vitro* genotoxicity testing are either derived from cancers or other malignancies, or are transformed cells with disrupted cell-cycle control.

Thus, by using combination of different GFP-reporter cell lines, we were able to discriminate between compounds that primarily induce genotoxic, oxidative stress or protein damage. With this study we demonstrate one example of a traditional *in vitro* assay to detect genotoxic properties of compounds, the comet assay, that doesn't have the same sensitive, and specific as the GFP-reporters, in which we were not able to detect a comet tail when cell were exposed to chemicals of the ECVAM 2 and 3, therefore with this study we developed a new *in vitro* assay that has an assay that is highly sensitive, and specific, and can provide insights in the relative toxicity potential of chemicals, is fast and allows us to reduce the number of mice used in bioassays.

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Supplementary data

Supplementary Method 1: BAC recombineering

Antibiotics:

Chloramphenicol (Cam) working concentration is 12.5 µg/ml

Tetracycline (Tet) working concentration is 3 µg /ml

Kanamycin (Kan) working concentration is 25 µg/ml

Gene construct

PCR mix:

Plasmid GFP-IRES-Neo	1 µL
Primer forward (10pmol/µL)	1.5 µL
Primer reverse (10pmol/µL)	1.5 µL
DNTPs (10mM stock)	1.5 µL
5x KAPA HiFi fidelity buffer	10 µL
KaPa HiFi polymerase	1 µL
H ₂ O	33.5 µL
Total	50 µL

PCR program:

98°C 5min	
98°C 30s	} 20x
55°C 30s	
68°C 2,50min	
68°C 10min	

Introducing pRedE/T vector into the BAC:

1. Inoculate 4 mL LC + Cam with one colony of the BAC O/N shaking at 37°C
2. Add 400 µL of O/N BAC culture to 40 mL LC + Cam, mix well and divide equally into 4 (50ml) falcon tubes (10mL each), incubate for 2 to 3 hours shaking at 37°C till OD₆₀₀~0,4.
3. Always keep the cells cold on ice, from now on.
4. Collect cell together and centrifuge for 10 min at 4°C (4000rpm)
5. Remove supernatant and wash pellet with 40 mL of cold 10% glycerol.

6. Centrifuge for 10 min at 4°C (4000rpm)
7. Remove supernatant, wash cell for a second time with 40 mL of cold 10% glycerol
8. Centrifuge for 10 min at 4°C (4000rpm)
9. Remove as much as possible from the supernatant
10. Add 100 µL of 10% glycerol to the cells, resuspend the electro-competent cells and add 50 µL to an eppendorf
11. Add 1 µL pRedE/T plasmid (20ng/µL) to the eppendorf with cells, mix by tapping, and leave on ice.
12. Electroporate cells: place cells in cold cuvette (1mm gap) and apply pulse (bacteria program 1.8kV, 200 Ω, 25uF)
13. Move cells quickly from cuvette with a Pasteur pipette and place into a tube with 1mL LC.
14. Incubate shaking at 30°C for ½ to 2 hours.
15. Plate 100 µL on LC + Cam + tet on 1 plate and the rest on another plate.
16. Incubate at 30°C O/N in the dark.

Introducing the IRES-Neo into the BAC:

17. Inoculate 4mL LC+ Cam + Tet with 10 colony of the BAC + pRedE/T O/N shaking at 30°C in the dark
18. Second day, add 400 µL of O/N BAC+ pRedE/T culture to 40mL LC + Cam+ Tet mix well and divide equally into 4 (50ml) falcon tubes (10mL each), incubate for 2 to 3 hours shaking at 30°C till OD₆₀₀≈0,2-0,3.
19. Collect cells together, add up to 0,2% of (10%) L-arabinose (800 µL for 40mL), mix well and divide equally into 4 falcon tubes (10ml each), incubate for 45min shaking at 37°C in the dark till OD₆₀₀≈0,4.
20. Collect cell together and centrifuge for 10min at 4°C (4000rpm)
21. Remove supernatant and wash pellet with 40mL of cold 10% glycerol.
22. Centrifuge for 10min at 4°C (4000rpm)
23. Remove supernatant, wash cell for a second time with 40ml of cold 10% glycerol
24. Centrifuge for 10min at 4°C (4000rpm)
25. Remove as much as possible from the supernatant
26. Add 100 µL of 10% glycerol, and add 50uL to an eppendorf
27. Add to 300ng of the gene construct.
28. Electroporate cells: place cells in cold cuvette (1mm gap) and apply pulse (bacteria program 1.8kV, 200 Ω, 25uF)
29. Move cells quickly from cuvette with a Pasteur pipette and place into a tube with 1mL LC.
30. Incubate shaking at 37°C for ½ to 2 hours.
31. Plate 100 µL on LC + Cam + tet + Kan on 1 plate and the rest on another plate.
32. Grow O/N at 37°C

Checking PCR on colonies

PCR mix:

Clone	
Primer forward (20pmol/ μ L)	0.5 μ L
Primer reverse (20pmol/ μ L)	0.5 μ L
DNTPs (2,5mM each stock)	2.5 μ L
5 x GoTaq buffer	5 μ L
GoTaq polymerase	0.1 μ L
H ₂ O	16.4 μ L
Total	25 μ L

PCR program:

94°C 5min	
94°C 30s	} 35x
55°C 30s	
72°C 1,5min	
72°C 10min	

Replate 2x and repeat PCR.

Supplementary Method 2: RNA analysis protocol

RNA isolation

1. Culture cells on gelatine with conditioned medium until well is full
2. Remove medium and wash 1x PBS
3. Add appropriate amount of TRIzol (Invitrogen) for the used culture well and incubate at room temperature for 5min
4. Add chloroform (40 μ L per 100 μ L TRIzol), shake for 15sec and incubate for 2-3minutes at room temperature
5. Centrifuge for 15min 12000 rpm at 4°C
6. Move water phase to new eppendorf, add ½ a volume of isopropanol and incubate for 10min at room temperature.
7. Centrifuge for 10min 1200 rpm at 4°C
8. Remove supernatant and wash pellet 1x with 100uL 75% Ethanol.
9. Centrifuge 10min 1200 rpm 4°C
10. Resuspend pellet in 20 μ L mQ and determine RNA concentration with NanoDrop

cDNA synthesis

11. Use 1 μ g of RNA and add mQ to a total volume of 9,5 μ L
12. Add 0,5 μ L oligo dt primer (12-18) and 4 μ L dntps (2,5mM each)
13. Incubate for 5min at 65°C and for 5min on ice
14. Add 4 μ L 5x first strand buffer, 1 μ L 0,1M dtt, 0,5 μ L RNazin and 0,5 μ L SuperScript reverse transcriptase III (Invitrogen)
15. Incubate for 1 hour at 50°C and for 10min at 65°C
16. GoTaq PCR
17. PCR Mix:

GoTaq buffer	5 μ L
dntps (2.5mM/each)	2,5 μ L
Sus 3 primer (Aprt forward)	0,5 μ L
Sus 4 primer (Aprt reverse)	0,5 μ L
GoTaq polymerase	0,1 μ L
H ₂ O	15,4 μ L
cDNA	1 μ L
Total	25 μ L

18. PCR program:

94°C 5min	} 35x
94°C 30s	
55°C 30s	
72°C 1,5min	
72°C 10min	

19. Load PCR product on 1% agarose TAE gel. Aprt will give a product of \pm 400bp

Supplementary Method 3: Comet assay protocol

Neutral Comet

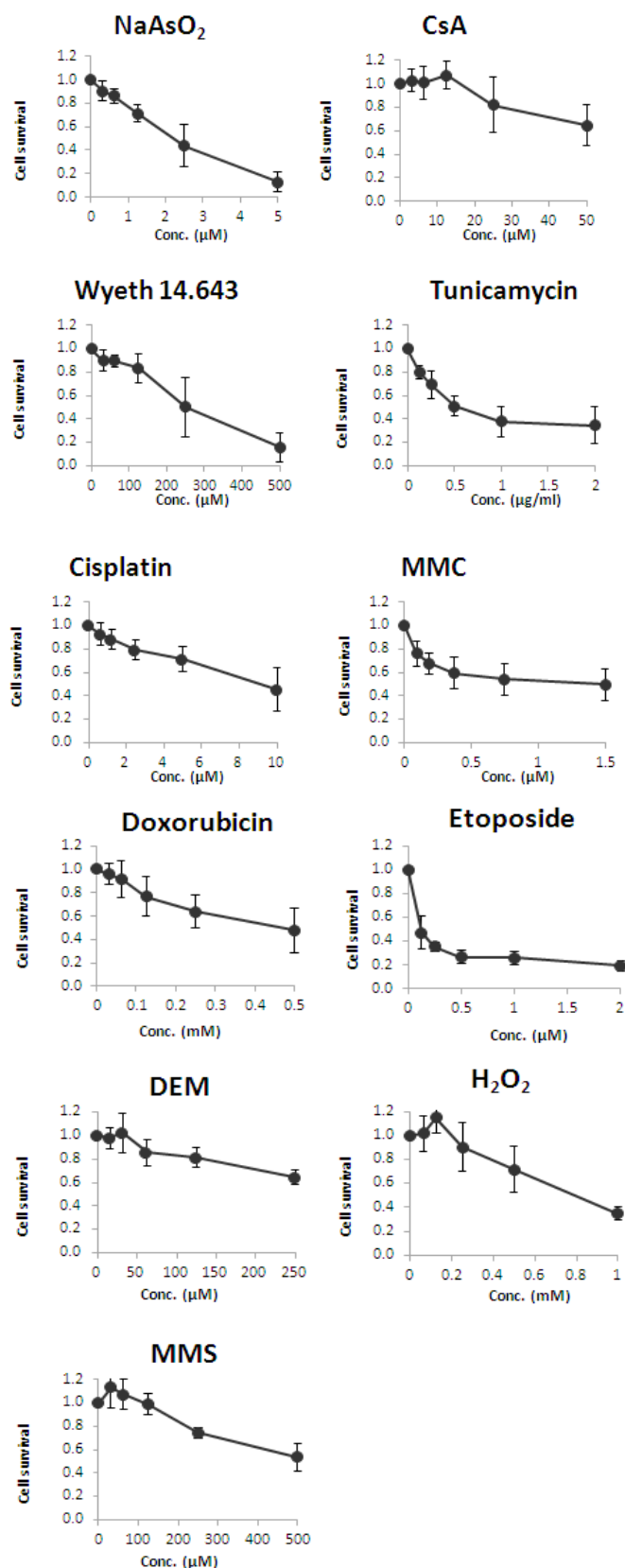
1. Cool down all of Lysis Solution and electrophoresis buffer
2. Treat cells with agents
3. Prepare 0.8% of LMA in PBS, cool down LMA at 37°C for at least 20min.
4. Trypsinise cells and resuspend with condition medium
5. Take 350,000 cells/ml to 1,5ml tube
6. Resuspend with 500µL PBS (Ca and Mg free)
7. Centrifuge (1000 rpm, 5 min)
8. Mix:
 - 1000 µL of pre-warmed LMA at 37°C
 - 100 µL cell suspension
9. Take 100 µL of mix, spread over the slides and immediately put the coverslip
10. Incubate in the dark at 4°C for at least 10min
11. Remove the coverslips (carefully)
12. Soak the slide in cold-lysis buffer for 1hr in the dark at 4°C
13. Wash slides 2 times with 1X cold- TBE, 5 min each
14. Run slides in fresh 1X cold-TBE at 1 Volt/cm for 20min at 4°C
(condition using electrophoresis chamber:
 - 2L 1X cold-TBE
 - 27V constant (mA will be about 5-7mA)
 - 20 min running)
15. Wash slides twice with water, 5 min each, in the dark at RT
16. Put slide in 70% Ethanol for 30min in the dark. Let them dry for overnight at RT.
17. Stain slides with SYBR gold for 30min in the dark, RT
(SYBR gold stock 10 µL + β-mercaptoethanol (2µM as final concentration) + 100mL TE)
18. Wash excess of dye with 100mL TE
19. Air-dry to allow slide dry completely, in the dark
20. Ready to use.

Alkaline Comet

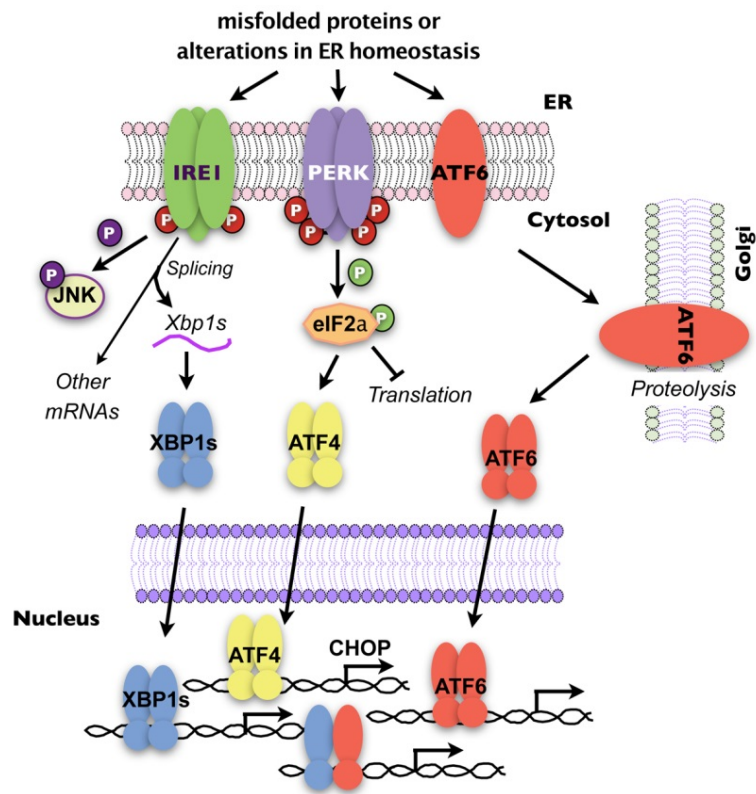
1. Cool down all of Lysis Solution and electrophoresis buffer
2. Treat cells with agents
3. Prepare 0.8% of LMA in PBS, cool down LMA at 37°C for at least 20min.
4. Trypsinise cells and resuspend with condition medium
5. Take 350,000 cells/ml to 1,5ml tube
6. Resuspend with 500µL PBS (Ca and Mg free)
7. Centrifuge (1000 rpm, 5 min)
8. Mix
 - 1000 µL of pre-warmed LMA at 37°C
 - 100 µL cell suspension
9. Take 100 µL of mix, spread over the slides and immediately put the coverslip
10. Incubate in the dark at 4°C for at least 10min
11. Remove the coverslips (carefully)
12. Soak the slide in cold-lysis buffer for 1hr at 4°C

13. Dip slides in unwinding solution for 30min at 4°C, in the dark
14. Run slides in fresh unwinding solution at 1 Volt/cm for 30min at 4°C
 - (condition using electrophoresis chamber:
 - 2L alkaline solution
 - 27 V constant (mA will be about 330-350mA)
 - 20 min running)
15. Wash slides 3x with 0.4M Tris pH7,5, 5 min each, in the dark at RT
16. Put slide in 70% Ethanol for 30min in the dark. Let them dry for overnight at RT.
17. Stain slides with SYBR gold for 30min in the dark, RT
 - (SYBR gold stock 10 µL + β-mercaptoethanol (2µM as final concentration) + 100mL TE)
18. Wash excess of dye with 100mL TE
19. Air-dry to allow slide dry completely, in the dark.
20. Ready to use.

**Positive control: mES B4418 exposed to 30min of 500 µM H₂O₂.



Supplemental fig. 1. Cell survival for all the tested compounds. The toxicity curve is based on the average for the four GFP-reporters. The cell survival was assessed after 24-h treatment by flow cytometry.



Supplemental fig. 2. The UPR sensors. PERK, IRE1 α , and ATF6 control mRNA translation and transcriptional induction of UPR-regulated genes. Upon accumulation of unfolded protein, PERK is activated by homodimerization and autophosphorylation to phosphorylate eIF2 α , thus reducing the rate of mRNA translation and the biosynthetic protein-folding load on the ER. eIF2 α phosphorylation increases translation of Atf4, a transcription factor that activates expression of genes encoding protein chaperones, ERAD machinery, enzymes that reduce oxidative stress. Dimerization of IRE1 α induces cleavage of Xbp1 mRNA. Then, Xbp1 mRNA is translated to produce XBP1s. ATF6 is transport to the Golgi where it's cleaved to release a transcriptionally active fragment. Cleaved ATF6 acts in concert with XBP1s to induce expression of genes encoding protein chaperones and ERAD machinery.

Supplemental table 1

Alkaline comet assay for all the tested compounds, as the treatment exposure, dose and the tail moment. The values are the mean ± SD of at least 50 cells. The compounds are divided in 4 classes: the ECVAM class 1, 2 and 3 and additional compounds. The higher tail moment for each compound is mark (*) and was used to make the Fig. 13. NA, not available due to too few cells or unscorable cell.

Chemical	Treatment (h)	Dose (μM)	Tail moment (mean±SD)
Untreated		0	0.0208±0.0182
ECVAM class 1			
Cisplatin	0.25	10	0.0525±0.032 *
	0,5		0.8545±0.26
	4		0.00015±7.32E ⁻⁰⁵
	6		6.92E ⁻⁰⁷ ±1.61E ⁻⁰⁷
	8		0.00032±0.0002
Methyl methosulfate	0,5	50	7.91±1.35
	0,5	100	9.54±1.41
	4	500	142.2±9.98 *
Cadmium chloride	4	10	0.0007±0.0007
		20	25.30±4.23 *
p-chloroaniline	4	1000	0
		2000	0
Sodium arsenite	0,5	5	6.52±2.10
	6	10	18.17±4.32
	4	20	91±12.01
	6		155.81±9.19 *
Taxol	1	1	0.00017±0.0002
	6	5	0.0104±0.0104 *
	6	10	0.0018±0.0018
Etoposide	0,5	1	4.83±0.84
		2	52.65±3.36
	1	2	145.26±4.93 *
ECVAM class 2			
Erythromycin stearate	4	5000	0.046±0.04
		10000	0.236±0.23 *
Hexachloroethane	4	5000	0.308±0.028
		10000	0.386±0.023 *
Methy carbamate	4	5000	0.455±0.27 *
		10000	0.008±0.01
Amitrole	4	5000	0.295±0.28 *
		10000	0.006±0.01
ECVAM class 3			
Eugenol	4	1000	0.010±0.01 *
		2000	1.37E ⁻⁰⁵ ±9.80E ⁰⁶
Propyl gallate	4	1000	0.092±0.03 *
		2000	0.025±0.01
nitrophenol	4	1000	0.005±0.004
		5000	0.165±0.11 *
2,3-dichlorophenol	4	1000	0.121±0.06 *
		2500	NA
resorcinol	4	1000	0.001±0.0004
		5000	0.053±0.05 *
sulfizoxazole	4	1000	0.005±0.004
		5000	0.162±0.12 *
Additional genotoxins			
Doxorubicin	4	0,5	0.290±0.023
	6	1	45.86±5.08
		2	149.91±7.23 *
Mitomycin C	0,5	1.5μg/mL	0.298±0.13 *
Diethly maleate	0,5	250	6.82±2.29 *
	0,25		246.94±7.25 *
	0,5		153.66±5.98
Hydrogen peroxide	1	500	36.834±5.12
	2		3.19±0.91
	4		2.207±0.92
Non-genotoxic carcinogens			
Tunicamycin	1	2μg/mL	0.0002±0.0002 *
	4		9.03E ⁻⁶ ±9.03E ⁻⁶
Cyclosporin A	6	25	0.012±0.01 *
		50	0.005±0.003